MAY 7 1941

American Journal of Veterinary Research

A Publication of the
American Veterinary Medical Association

Volume II APRIL 1941 Number 3

Consecutive order of appearance, rather than identity with any given volume, is the plan adopted for numbering the issues of American Journal of Veterinary Research. The introductory issue, published in October 1940, thereby became Number 1; the second issue, January 1941, Number 2; and the present issue, April 1941, Number 3.

Volume I consists of but one issue (October 1940, Number 1). The January 1941 issue (Number 2) marks the start of Volume II.

COPYRIGHT 1941 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

Bull Lit

CONTENTS

Research in Nutrition: Its Contribution to Livestock Production (Editorial by George H. Hart) 131

Pox in Swine—L. H. Schwarte and H. E. Biester..... 136

A Preliminary Report on the Effect of Colloidal Silver Oxide on Bovine Mastitis—F. J. Weirether, E. O. Anderson, R. E. Johnson, W. N. Plastridge and E. Jungherr..... 141

Effect of Novoxil Liquid on Catarrhal (Chronic) Mastitis and on Streptococcus Agalactiae Udder Infection—Louis A. Klein, Albert L. Kleckner and Robert O. Biltz..... 145

Studies on Control of the Liver Fluke of Cattle in the Hawaiian Islands—Joseph E. Alicata 152

The Hereditary Transmission of Anaplasmosis by Dermacentor Andersoni Stiles—D. E. Howell, Geo. W. Stiles and Lewis H. Moe..... 165

The pH of Vaginal Mucus of Normal and Sterile Dairy Cows—Sedgwick E. Smith and S. A. Asdell..... 167

Enteritis in Sheep Caused by Infection with the Protozoan Parasite, Globidium Gilruthi—H. Marsh and E. A. Tunnicliff..... 174

Results Obtained by Long-Continued Cultivation of Brucella Abortus in Living Chick Embryos—H. J. Metzger and Freida R. Stokes..... 178

Bacterial Dissociation in Brucella Abortus—C. K. Mingle and C. A. Manthei..... 181

Studies on Certain Filtrable Viruses. III. Longevity of Chorioallantoic and Comb-Tissue Fowl-Pox Virus in Vitro—C. A. Brandly, Robert Graham and C. C. Morrill..... 190

Preliminary Studies on the Blood Chemistry of the Fox—E. H. Spitzer, A. I. Coombes and W. Wisnicky 193

Data and Bibliography on Some Nitrogenous Constituents of Normal Dog's Blood—James B. Allison, Herman O. Dreskin and M. L. Morris..... 196

A New Record of a Deer Parasite for California—R. F. Annereaux..... 199

Swine Erysipelas in Turkeys—A. S. Rosenwald and E. M. Dickinson..... 202

Macroscopic Differentiation of Lesions of Histomoniasis and Trichomoniasis in Turkeys—Ena A. Allen..... 214

Studies of Chick-Embryo-Propagated Equine Encephalomyelitis Virus and Vaccine: Antigenicity and Preservation—M. S. Shahan and E. A. Eichhorn..... 218

Studies on Equine Encephalomyelitis—F. M. Birch..... 221

The Determination and Clinical Correlation of Variations in the Calcium, Inorganic Phosphorus, and Serum Proteins of Horse Blood—A. Henry Craige, Jr., and John D. Gadd 227

Announcement 256

The American Journal of Veterinary Research is published quarterly by the American Veterinary Medical Association, 600 S. Michigan Ave., Chicago, Ill.

Editorial Staff

L. A. MERILLAT and J. G. HARDENBERGH, Editors

J. J. SHAFFER, Assistant to the Editors

Committee on Journal

A. E. WIGHT, I. E. NEWSOM, H. W. JAKEMAN

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Subscription price: $4.00 per year for United States, Canada, and countries affiliated with the Pan American Postal Union; foreign countries, $4.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $5.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

Consecutive order of appearance, rather than identity with any given volume, is the plan adopted for numbering the issues of American Journal of Veterinary Research. Volume I consists of but one issue (October 1940, Number 1). The January 1941 issue (Number 2) marks the start of Volume II.

American Journal of Veterinary Research

VOL. II APRIL 1941 NO. 3

Research in Nutrition: Its Contribution to Livestock Production

DEVELOPMENT of knowledge does not usually progress on an even keel. After more or less rapid advance in any field it may mark time for a generation or even stagnate while waiting for new concepts to be presented. When this occurs, activity develops and great strides forward may be made over a period of years, bringing knowledge in the particular subject far ahead of its place when the stimulus was applied. Nutrition at the present time offers an outstanding example of this general rule.

At the close of the last century research in nutrition was not active. It was then quite generally believed that a complete diet consisted of carbohydrates, fats and proteins. These were so proportioned in rations as to provide a proper nutritive ratio, narrow for growing and lactating animals, and wider for fattening animals. Palatability and coefficients of digestibility were given consideration to insure an intake of sufficient quantity for maintenance and production. All feeds contained inorganic elements, termed ash, and these with the addition of salt and some consideration of fiber content constituted the problem of nutrition for our farm animals. Work on this phase of the animal-husbandry field largely consisted of feeding trials to determine the value of one feed in a ration as compared with another, or a mixture of feeds as compared with commonly fed materials constituting a basal ration.

Bacteriology was in a state of feverish activity, with great progress having been made for some years by virtue of the concepts of Pasteur. Genetics was just about to enter into an epoch-making period on increased knowledge of heredity through the rediscovery of Mendel's law of ancestral inheritance, made in 1866 but unrecognized until 1900 when it became independently rediscovered by De Vries in Holland, Correns in Germany and Tschermak in Austria.

In nutrition, research rumblings soon to become of world-wide interest were occurring. As examples of this, among many others, might be mentioned the work of Takaki on beriberi in the Japanese navy about 1882 and particularly that of Eijkman on polyneuritis in fowls and beriberi in the Dutch East Indies in 1897.

Under practically all conditions it is the work of many men and various incidents in different countries that lead up to the presentation of scientific concepts of international importance. Many give Hopkins of Cambridge University the credit of ushering in the new doctrine on nutrition when in 1906 he stated: "No animal can live upon a mixture of pure protein, fat, and carbohydrate, and even when the necessary inorganic material is carefully supplied the animal still cannot flourish."

In 1911, the cure of polyneuritis gallinarium with small quantities of a substance obtained from rice bran was demonstrated by Funk. He was the first to give this necessary accessory food factor the name beriberi vitamin. It is now known that this is the heat-labile fraction of the water-soluble B complex known as B_1. It is now prepared synthetically under the name of thiamin chloride. The period from this finding by Funk to the present time marks

the discovery of new vitamins and their chemical synthesis, and the finding of abnormalities in animals caused by their lack and how to administer them to whole populations of human beings and domestic animals.

DIETARY ESSENTIALS IN LIVESTOCK

The livestock industry for a long time looked upon the new discoveries in animal nutrition as an academic matter having little or no practical application. This was logical because of the constant and widespread efforts at exploitation with proprietary livestock feeds and nostrums. Those interested in profit from this exploitation know that the time to make hay is when the sun shines. The sun shines for them in the field of nutrition when knowledge is incomplete and yet far enough along that widespread public interest exists. There is a principle involved in this which should be manifest at all times to those dealing with livestock problems: This is the recognition of the difference between medication and nutrients applied to livestock management. Medication is applied to individual animals for short periods while they are sick or to the mass treatment of flocks and herds in the form of vaccination once a year or once during their lives. Price is always important, but costly medication is economically feasible. On the other hand, food essentials must be supplied throughout the life of the animals, and most of them every day.

It has been said that the value of a food essential depends upon the need of the animal for that particular substance. Thus it may approximate the value of the animal, because without the essential substance death will result. This does not mean that it is economically advisable, except possibly in the case of pet animals and Thoroughbred horses, to pay excessive prices for specially prepared small fractions of the diet. Rather, it must be recognized that average feeds contain the essential dietary ingredients. This gives credence to the importance placed on variety in animal feeding. Old husbandry recommendations called for the rations to be made up from at least five different plant sources. With the basic principle in mind that normally used feedstuffs in the great majority of cases supply the essential nutrients, we are prepared to look calmly into the contributions which research has made to livestock production.

RESEARCH FINDINGS

It is known that all species of farm animals are not alike in their requirements. For example, the work of Dutcher, McElroy and Goss, Bethke, Pearson and the Wisconsin group has shown that in cattle and sheep the various factors in the water-soluble B complex, including thiamin, riboflavin, pantothenic acid, nicotinic acid and pyridoxine, are synthesized by bacterial action in the rumen. It is not necessary for these substances to be in the diet of these animals. It is probable that their synthesis also takes place in the cecum of the horse, but no experiments have been carried out to prove it. On the other hand, synthesis of these factors does not occur in the pig and this species must have the B complex in its diet. Sir Charles Martin and coworkers in England and E. H. Hughes in this country have shown deficiency symptoms in this species due to lack of factors in the B complex which became so serious under some conditions as to affect the economic stability of a swine-production enterprise.

The B complex is important in poultry and has to do with the hatchability of eggs and the livability of chicks. Vitamin C, which is ascorbic acid, when given subcutaneously has recently been demonstrated to be of value in temporary sterility in bulls. Vitamin A is essential in all species of farm animals and a variety of abnormalities result from its lack in the diet after body storage has been exhausted. Vitamin E deficiency, on the other hand, has not been observed in domestic animal populations with the possible exception of poultry maintained on highly synthetic experimental rations, and the work of Thomas of Iowa with goats would seem to be conclusive in rendering its natural occurrence

very doubtful. This means that it is widely present in ordinary feedstuffs or synthesized in the bodies of domestic species.

Research has shown that a variety of minerals are essential in animal nutrition, and species differences exist regarding susceptibility to lack of certain mineral elements. Thus, animals living largely on roughages, as cattle and sheep, eat the leaves and stems of plants and these are usually rich in calcium. Hogs and poultry, however, being capable of handling roughage in only a limited way, live largely on the seeds of plants which are rich in phosphorus. These two elements are usually considered together because the great storehouse of them in the body is the skeleton, where they are deposited together as tricalcium phosphate.

When minerals are supplied to livestock beyond that which is contained in their regular feeds, hogs and poultry are given calcium in the form of ground limestone or oyster-shell flour because this is the cheapest source of calcium. Cattle and sheep are supplied with bone meal mainly because this is the cheapest source of phosphorus. It should be remembered that bone meal contains about 32 per cent of calcium against 14 per cent of phosphorus and, therefore, no additional calcium is required when bone meal is supplied. Deficiency of calcium in farm animals other than poultry and swine is now recognized to be of rare occurrence. Dairy cows are usually in negative calcium balance during heavy milking, but regain normal body stores during the latter part of the lactation and in the dry period.

Twenty-five years of research were required to explain why injecting air into the udder of the dairy cow cured milk fever. Then it was found that the symptoms of milk fever resulted from too low a level of calcium in the blood and calcium gluconate became the nutritional or therapeutic agent to relieve the trouble. The symptoms do not usually result from too low a calcium content of the body but, rather, the temporary inability of the animal to marshal calcium into the blood from the body stores as rapidly as it is being taken out in the milk of heavy producers. Further research is needed to more fully explain this phenomenon. Parathyroid gland activity is involved, because this is the ductless gland which functions to regulate the blood-calcium level.

Research has shown further that iodine is essential and that widespread areas exist where it is deficient. In some of these areas symptoms of its deficiency are much more prevalent in the human than in the animal population. Some evidence of deficiency may exist in a given area while in closely adjoining areas no manifestations are evident. Investigators are still wrestling with these peculiarities in the hope of finding an explanation.

Nutritional studies have shown that anemia in young nursing pigs kept on wood or cement floors in the control of intestinal roundworms is overcome by painting the udder of the nursing sow with iron preparations.

Also, on the white and gray sandy soils in Florida, extensive research by Becker, Neal and Shealy has shown a form of anemia in cattle in which the hemoglobin content of the red blood corpuscles is reduced and the animals fail to develop normally, emaciate and finally die if left too long on the deficient areas. The condition is due to a combined deficiency of iron and copper.

A vast amount of other similar work with vitamin and mineral deficiencies could be cited from this and other countries.

Incidence of Identical Affections in Widely Separated Parts of the World.—In the early development of new knowledge of nutrition it was the common practice to describe abnormalities of livestock under a local name applicable to the particular part of the world where the condition existed. These local terms still confuse the literature to a considerable extent. Thus, in phosphorus deficiency a great variety of local names were applied because it is a condition occurring widely over the surface of the earth. Among the names applied to the condition, or conditions thought to be caused by it, are bone chewing, loin disease and creeps in Texas, lambsiekte and styfsiekte in South Africa, bulbar paralysis, stiffs, wobbles, pegleg disease, coastal disease, Midland disease, Winton disease in

Australia, Waihai disease in New Zealand, Hinsch disease in the Black Forest in Germany, and rickets and osteomalacia as descriptive of the bone alterations.

The work of Orr at the Rowett Institute, Scotland, in gathering information on these conditions in all parts of the far-flung British Empire and in other countries resulted in the publication of his book in 1929 on *Minerals in Pastures* which greatly helped to correlate existing knowledge on the subject at the time.

HEREDITY AND ENVIRONMENT

It became self-evident that all parts of the world did not supply an environment of equal value for the raising of high-quality livestock. Any animal is the sum of its inheritance and its environment. If the environment is too rigorous to allow the full expression of the qualities of its germ plasm, then it may be said to have been bred beyond its environment.

Hammond of Cambridge showed this to be the limitation of livestock improvement on the island of Trinidad, where one cross of native cattle with improved foreign breeds was as far as it was economically sound to proceed. Thus, it became evident that the mere shipping of high quality purebred livestock from the lush pastures of England and Scotland known as the cradle of the breeds to distant parts of the Empire did not necessarily mean improvement in the livestock of the area to which they were sent for breeding with the native stock.

It remained for Bisschop to elucidate this problem conclusively in large-scale experiments with cattle begun in 1925 at the Armoedsvlakte research station near Vryburg in British Bechuanaland and reported at the 13th International Veterinary Congress at Zurich in 1938.

In South Africa there was a large, indigenous cattle population consisting of numerous types, subtypes and breeds which had existed in their respective habitats for many generations. They were so-called native or scrub cattle but were in harmony with the environmental complexes in which they were originally found by the white man. Attempts at selective breeding had been made with only one of the subtypes, the Africaner breed, up to the beginning of the present century. The work of Theiler, Green and du Toit had shown that deficiency of phosphorus in the soils and natural pastures was the direct cause of inferior growth, production and reproduction in cattle in the western parts of the Union of South Africa. The experiment consisted in cross breeding groups of native cows with bulls of improved breeds. Each group of females was subdivided into two herds, one of which received phosphorus supplement in the form of bone meal. Otherwise the animals subsisted entirely on the vegetation of the open range. The bulls consisted of the Africaner representing the indigenous breed. Three exogenous breeds were used: the Sussex for beef, the Redpoll for dual purpose and the Holstein-Friesian for dairy.

The work has shown that without phosphorus supplement attempts to improve the native cattle were a complete failure. With this single and inexpensive supplement the Africaner breed were able to maintain a satisfactory standard of excellence in their offspring from native cattle. On the other hand, this could not be done with the exogenous European breeds because further limiting environmental nutritional factors on the veldt still caused deterioration. This became evident in the second and third generation ¾ and ⅞ blood crosses of the native animals with the improved exogenous breeds. It was concluded that to import breeding cattle to any country from markedly superior environments is to court disaster. The safer policy is to breed selectively within the indigenous population up to the maximum degree of excellence attainable under the prevailing environment. If the environmental level can be raised by improving the feed supply, then the limits of selective breeding can be proportionately extended. Permanent breeding herds should always be bred up to and never down to environmental limits. Environment in animals is largely management, mainly consisting of nutrition. The data from this experiment explain the situation that frequently exists on poorly managed

livestock farms in relatively good feed areas.

Meeting Deficiencies in Local Cheap Feeds

Nutrition is making real contributions to livestock production in showing how to make the most economical use of locally produced feeds, which are usually cheap, by supplementing their deficiencies rather than transporting from considerable distances relatively high-priced, well-known feedstuffs. The following may be mentioned as examples:

In Kansas it was shown by McCampbell that the over-all value of a ration containing prairie hay plus calcium carbonate and grain mixture was nearly equal to alfalfa hay and grain mixture at much less cost. In Utah the same was shown by Maynard for alfalfa and beet pulp plus bone meal as compared with the more expensive alfalfa and beet pulp plus cottonseed cake, because it was the phosphorus in the cake that was required and not the additional protein.

In California, Guilbert showed that the over-all value of grain hay, cottonseed meal and barley was made much greater at no extra cost by replacing part of the grain hay with more expensive alfalfa and part of the cottonseed with less expensive beet pulp mainly because vitamin A in the alfalfa was needed to cure night blindness and diarrhea in the fattening animals. Today this could be done by adding a few drops daily per animal of high vitamin A-potency shark liver oil to the concentrates.

Urea a Source of Protein for Ruminants

Many years ago it was ascertained in Germany that ruminants could utilize urea as a source of nitrogen in the diet. Urea is a waste-end product of protein metabolism eliminated in the urine. Ruminants are able to utilize this product because the bacterial action in the rumen probably converts the urea into amino acids within the bacteria. These are subsequently digested in the fourth stomach and intestines and thus can supply part of the protein requirements of these animals.

Recently a process for developing a relatively cheap and abundant source of urea was perfected in this country and investigations at the Wisconsin, Illinois and Massachusetts agricultural experiment stations have demonstrated that it can be used as one source of protein in feeding cattle and sheep.

The nitrogen content of proteins is about 16 per cent and the protein content of a feed is determined on its nitrogen content multiplied by 6.25. Urea, a non-protein, contains 46.5 per cent of nitrogen. If this were all converted, it would produce upwards of three times its weight of protein. Thus, nitrogen in the form of urea could replace a much larger quantity of nitrogen in the form of protein, and this would probably make urea considerably cheaper than most protein feeds on the basis of its protein value.

A discovery of this kind at the present time is of minor importance on the Pacific Coast where protein feeds are relatively cheap. It may, however, become of great value in other places, for example in the Hawaiian Islands. There energy-producing feeds, such as molasses and pineapple pulp, are in great overabundance, but protein feeds are scarce. The latter have to be shipped in from long distances at great cost.

Veterinarians Should Keep Informed

To be familiar with recent discoveries and constantly developing knowledge in nutrition is important to the veterinary profession. We are constantly in an advisory capacity on the health and well being of the domestic animal population. To recognize abnormalities of nutritional origin along with those from other causes will make us masters of situations where hopeless confusion will exist without such knowledge. Nutritional deficiency conditions are of more common occurrence than formerly believed. A balanced diet containing all the essential ingredients is necessary for the well being of farm animals and the economical production of animal products. [*Editorial contribution by George H. Hart, D.V.M., College of Agriculture, University of California, Davis, Calif.*]

Pox in Swine*

L. H. SCHWARTE, B.S., M.S., D.V.M., Ph.D., and H. E. BIESTER, V.M.D.
Ames, Iowa

THE OCCURRENCE of pox in swine was reported as early as 1842 by Spinola[1] and was believed for many years to be closely related to vaccinia. The existence of a primary swine pox has been considered doubtful. As the result of the investigations carried on during the past decade, a better understanding has been reached regarding the etiologic agent and the variations found under both field and experimental conditions.

PREVIOUS INVESTIGATIONS

It has been definitely shown that the pathologic changes in swine characteristic of pox may be produced by two different viruses. Nearly all of the cases studied by Akazawa and Matsumura[2] and by Yoshikawa[3] were associated with virus that was immunologically similar to that of vaccinia. Akazawa and Matsumura were able to establish the virus in rabbits. Yoshikawa reported favorable results by immunization with vaccinia virus. Manninger[4] and his associates reported a case of pox in swine from which vaccinia virus was isolated. However, other workers as well as Manninger also describe a disease of swine characterized by similar pox lesions caused by a filtrable virus immunologically different from vaccinia virus. This virus, unlike that of vaccinia, can not be transmitted to rabbits. The gross and microscopic pathology of the pox lesions produced by these two viruses are similar. The typical lesion produced at the height of its development in swine consists of an area of elevation on the skin with a brownish or black crust in the center which is often depressed or umbilicated. However, in some instances the lesions may be elevated, showing no central area of depression. The rapid spread of this infection in the swine herd has been attributed to lice. Pen exposure in the absence of lice was not followed by a spread of the disease. The virus remains viable in the bodies of lice kept under refrigeration for 3½ months.

"SWINE POX" WITH REFERENCE TO TERMINOLOGY

In 1940, Manninger *et al* proposed that the disease in swine which is produced by the virus immunologically similar to vaccinia virus be designated as "swine pox," whereas the identical pathologic condition produced by the filtrable virus which is immunologically distinct from that of vaccinia be designated as "pox-like eruptions of swine." It appears to us at the present time that absence of an immunologic relationship of this virus to vaccinia virus should not preclude designating this condition as swine pox. Apparently, the virus accepted as the cause of avian pox has no immunologic relationship to that of vaccinia and this does not invalidate the designation of fowl pox. For this reason, we are continuing the use of the term "swine pox" for the disease produced by the virus which is immunologically distinct from vaccinia virus.

OBSERVATIONS ON AN OUTBREAK OF SWINE POX

A rather unusual outbreak of this disease occurred on a large swine farm during the month of September 1940. Similar outbreaks have been reported about the same time on other farms. The disease was confined to suckling pigs ranging in age from 4 to 6 weeks. The lesions were confined to the back and sides of the affected animals. The lesions, the majority of which measured approximately 1 cm. in diameter, were round and rather smooth with no central areas of depression. The margins of the lesions appeared light gray, similar to those observed in some fungus infections (fig. 1). Shortly after the maximum development of the lesions, dark brown crusts formed. Considerable skin irritation was apparent during the course of the disease. As the result of scratching and rubbing on the wire fences, the crusts became cracked and broken, allowing a serous exudate to be liberated. Flies and insects were attracted by this

*From the Veterinary Research Institute, Iowa State College.

[1]Spinola: Krankheiten der Schweine (1842), p. 204.

[2]Akazawa and Matsumura: Rpt. Govt. Inst. Vet. Res. (Fusan), x (1937), p. 8.

[3]Yoshikawa: J. Jap. Soc. Vet. Med., ix (1930), p. 2.

[4]Manninger, Cosontos and Salyi: Arch. f. Tierheilk., lxxv (1940), p. 12.

exudate, thus causing secondary infection of the lesions and apparently spreading the infection to other susceptible animals in the immediate vicinity. No lice were found on the pigs which were observed at this time. The swine were confined in units approximately one acre in size. The infection spread rapidly to four of these units in one section of the farm. Later the disease appeared in two units about one-quarter mile from the original focus of infection. The animals in the other units were not affected. The infection spread rapidly until the arrival of cold weather, which arrested the activity of the flies and mosquitoes. The course of the disease was about six weeks in most cases, recovery being delayed by secondary infection. The growth and development of the diseased animals were seriously retarded but the mortality was negligible.

TRANSMISSION EXPERIMENTS

Isolation of Virus.—Suitable material was obtained from the lesions of the diseased animals which appeared to be free from secondary infection. This material was finely triturated in a mortar to which sterile physiological saline solution was added. A dilution of 10^{-3} was made for filtration through Berkefeld filters. Bacteria-free filtrates were used for animal inoculation as well as the crude material secured from diseased animals.

Animal Inoculation.—Vigorous young pigs approximately 8 weeks of age were selected for experimental inoculation. Large areas of the skin on the back and underline were thoroughly cleansed following the removal of the hair. Inoculations were made by means of needle punctures and scarification. Crude infectious material and filtrates passed through Berkefeld "V" and "N" filter candles were used. Positive reactions were secured in all inoculated pigs.

Several days after the experimental inoculation, a mild general reaction was observed following a slight rise in temperature. On the fourth and fifth days, small red spots appeared at the point of inoculation. On the back and sides of the inoculated animals the lesions developed rapidly into smooth, hard elevations ranging from 1 to 3 cm. in diameter. Hard crusts formed on the surface of these lesions which soon cracked and dropped off. On the underline the small eruptions developed into typical pox lesions, passing through the successive stages of papule and vesicle formation, becoming umbilicated and covered with dark brown crusts (fig. 2). These crusts dropped off in several days and complete recovery followed in most instances in 12 to 14 days. Subsequent inoculation of recovered animals failed to produce any reaction, indicating a substantial immunity.

Inoculation into Other Species. — Experimental inoculations were made in various species of animals, shown in table I, to determine the susceptibility of these animals to this virus infection. Cutaneous inoculations were made, the same technic being used that was employed in the transmission of this disease to susceptible pigs. The first inoculations were made with the Berkefeld "N" filtrate. The pig was the only species in which a positive reaction was observed. Two weeks after the first inoculations were made, these same animals were again inoculated with crude unfiltered virus. No reactions resulted from these inoculations. The inoculations in man were confined to the Berkefeld "V" and "N" filtrates because of the danger of bacterial infection from the crude virus suspension.

TABLE I—Animal Inoculation Experiments with Original Swine Virus

SPECIES	REACTION
Horse	—
Calf	—
Sheep	—
Pig	+++
Dog	—
Cat	—
Domestic fowl	—
Rabbit	—
Guinea pig	—
Rat	—
Mouse	—
Man	—

RELATIONSHIP OF SWINE-POX VIRUS TO VACCINIA VIRUS

It is known that various species of animals are more or less susceptible to vaccinia infection, especially the rabbit, which is highly susceptible to this disease. Because none of these species, with the exception of the pig, reacted to inoculation with the swine-pox virus, it was advisable to determine whether or not these animals were immune to vaccinia. The same experimental animals previously inoculated with the swine-pox virus, including two pigs which had recently recovered from the swine infection, were inoculated cutaneously with vaccinia virus. Table II indicates the results obtained from this procedure.

Strong positive reactions were secured in the horse, calf, pigs and rabbits inoculated with vaccinia virus. The reactions in the sheep and dog were definitely positive, but not nearly as severe as those of the other animals. Since the experimental animals which were refractory to infection with

TABLE II—Relationship of Swine-Pox Virus to Vaccinia Virus in Experimental Animals

Experimental Animal	Swine Virus		Vaccinia Virus	
	Inoculation Date	Reaction	Inoculation Date	Reaction
Horse	9–29–40	—	11–7–40	+++
Calf	10–17–40	—	11–7–40	+++
Sheep	9–29–40	—	11–7–40	+
Pig 1	9–20–40	+++	10–4–40	+++
Pig 2	9–20–40	+++	10–4–40	+++
Dog	9–29–40	—	11–7–40	+
Rabbit 1	9–20–40	—	10–4–40	+++
Rabbit 2	9–20–40	—	10–4–40	+++

the swine-pox virus proved to be susceptible to that of vaccinia, it would indicate that these viruses are immunologically distinct. Furthermore, the positive vaccinia reaction in the pigs which had recently recovered from swine pox indicates that there was no immunity or protective influence developed against vaccinia infection. However, pigs recovering from swine-pox infection proved to be immune to subsequent inoculation with swine-pox virus. From the experimental evidence presented, it seems that there is no immunologic relationship apparent between this virus and that of vaccinia.

Pathology

The lesions in the field cases were confined to the back and sides of the diseased animals. Following a hyperemia and lymphatic distention, the early lesions indicated that the epidermis had undergone a proliferation to form definite epithelial papules. This proliferation occurred not only upward but downward, involving the stratum granulosum. The degenerative process was characterized by vacuolization and ballooning of the cells in the papillary and reticular strata (figs. 3 and 4).

However, the degenerative process did not reach proportions usually encountered in the typical pox lesions. The superficial necrotic tissue developed into smooth gray-ish-brown crusts which in many instances became cracked, exuding serous exudates.

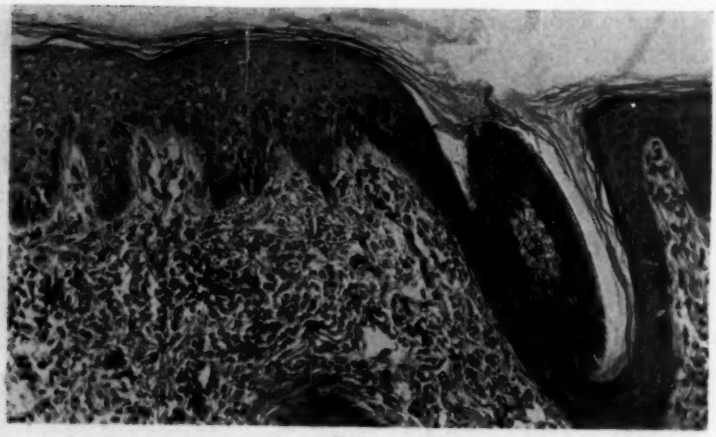
The stages of vesiculation and pustulation were not as well defined as commonly encountered in pox lesions. Regeneration of the epithelium accompanied by desiccation of the necrotic tissue with crust formation was rapid (fig. 5). Figure 6 shows completion of epithelial regeneration. Secondary infection was accompanied by extensive leucocytic infiltration. More extensive necrosis was observed and the regeneration of the epithelial structures was seriously retarded.

In the experimentally induced cases, the lesions on the back were comparable with those described from field cases. However, these experimental animals were confined to clean pens in a screened building, comparatively free from insects. Secondary infection of the lesions produced by virus filtrates was eliminated. The lesions produced on the underline of the experimental animals were not characterized by an extensive cellular proliferation. The localized hyperemia and congestion were more evident. The vacuolization and distention of cells seemed to be greater. The stages of vesiculation and pustule formation were more marked. Desiccation seemed to take place first in the center of the lesion causing a definite umbilication, typical of true pox lesions. The regeneration of the epithelium

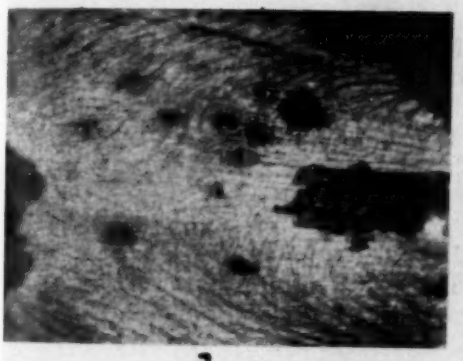
CAPTIONS TO ILLUSTRATIONS ON OPPOSITE PAGE

1) Typical lesions on the back of a pig, taken from a field case.
2) Lesions on the underline of experimental pig.
3) Inflammatory reaction and early stage of vacuolization (x 150).
4) Vacuolization of cells and leucocytic infiltration (x 300).
5) Area of degeneration with superficial crust formation (x 150).
6) Later stage of epithelial regeneration (x 150).

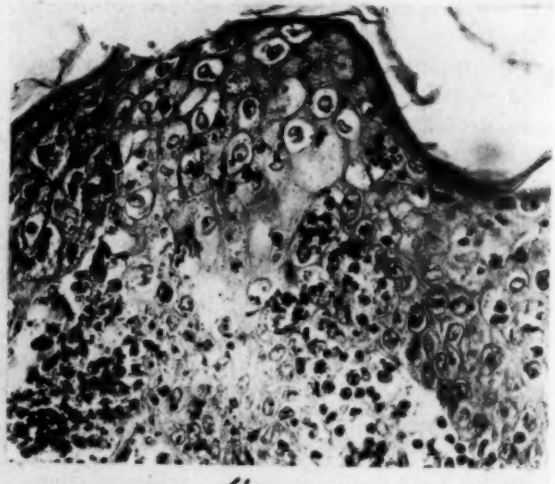
1



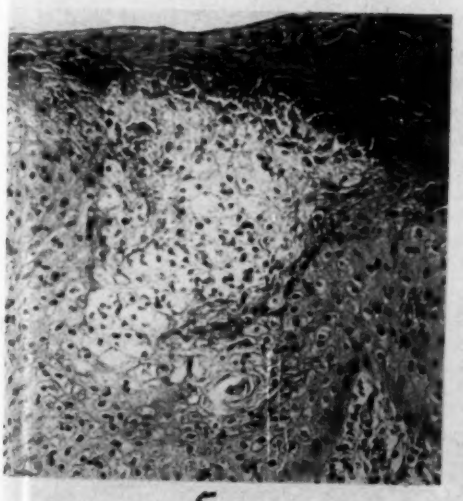
3



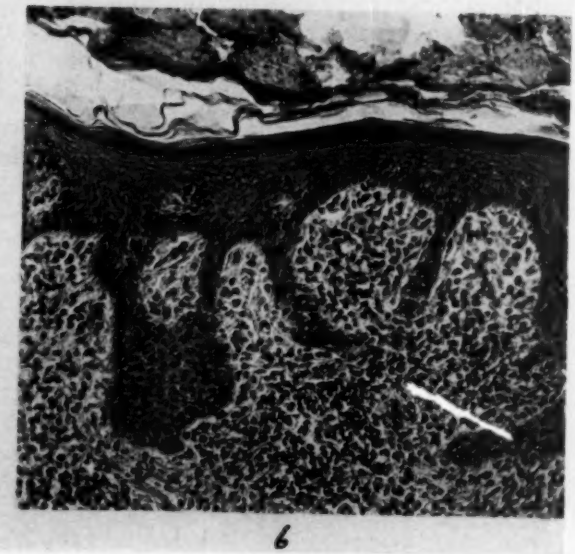
2



4



5



6

was more rapid than that on the back of the animal. Undoubtedly, this was due to the comparatively greater vascularity of the skin on the underline of the pig.

DISCUSSION

The gross appearance of the lesions which occurred only on the back and sides of the suckling pigs under field conditions was confusing to the diagnostician. The rapidity of the spread of this infection to swine which were free from external parasites might be considered rather unusual because this disease has been considered as being spread through the medium of the louse (*Hematopinus suis*). The sudden inhibition in spread and more rapid recovery following the inactivation of the insects by cold weather seemed to indicate that the spread of this infection was due to insects other than the louse.

The transmission of this disease by bacteria-free filtrates producing lesions similar to those of the field cases on the backs of the experimental swine and lesions typical of the umbilicated pox lesions on the underline of these animals indicates the wide variations possible in the gross appearance of the lesions produced. However, the histopathologic examination of these different types of lesions showed the same general pathologic changes, including the initial inflammatory reaction followed by degeneration of the epithelial cells, later infiltration with leucocytes and finally the epithelial regeneration which are considered typical of true pox lesions.

The failure to transmit this disease to other species clearly indicates the specificity of this condition to swine. This virus of swine pox is immunologically distinct from that of vaccinia and is in accord with the report of Velu,[5] who found rabbits refractory to infection with swine-pox virus. Manninger[4] and his associates distinguished two types of pox infection in swine, one very closely related to, if not identical with, that of vaccinia, and one immunologically distinct. The course of the disease and recovery without serious losses is similar to the swine pox described by Glässer.[6] The nature of this virus and characteristics of this disease, together with the similar experiences previously reported by others, are sufficient to justify the use of the term "swine pox" for the disease produced by the virus which is immunologically distinct from that of vaccinia.

SUMMARY

An outbreak of pox in swine showing a rather unusual type of lesion has been described. The virus was isolated and the disease was transmitted to susceptible pigs. Experimental transmission of the disease to animals other than swine was unsuccessful. The pathologic changes observed on the back of the experimental swine resembled those found in the field cases while those produced on the underline were typical of those caused by pox infections. Under field conditions the disease was apparently spread by insects other than the louse. The symptoms of the disease were mild and the mortality was very low. The virus is immunologically distinct from that of vaccinia. Recovery from this disease produces immunity against subsequent infection but does not immunize against infection from vaccinia virus. This condition is designated provisionally as swine pox.

[5]Velu: Rec. Méd. Vét., xcii (1916), p. 24.

[6]Glässer: Die Krankh. des Schweines (M. & H. Schaper, Hanover, 1927), p. 276.

A Preliminary Report on the Effect of Colloidal Silver Oxide on Bovine Mastitis*

F. J. WEIRETHER, B.S., E. O. ANDERSON, M.S., R. E. JOHNSON, M.S.,
W. N. PLASTRIDGE, Ph.D., and E. JUNGHERR, D.M.V.

Storrs, Conn.

DURING the past eight years, results obtained by us[1, 2] and by Minett, Stableforth and Edwards[3, 4] have shown that bovine mastitis which is caused by *Streptococcus agalactiae* may be controlled by segregation on the basis of periodic bacteriologic tests, and gradual replacement of infected animals with first-calf heifers raised on the farm. However, the less common forms of mastitis caused by staphylococci, streptococci other than *S. agalactiae* and coliform organisms have not been completely eliminated by the segregation and disposal method. The finding of a means of eliminating mastitis organisms from the udder would be a valuable supplement to the testing and segregation program in the control of mastitis caused by organisms other than *S. agalactiae*, and possibly in salvaging some animals infected with *S. agalactiae*. The possible use of colloidal silver, as prepared by Muller,[5] in overcoming udder infection with different mastitis organisms is being investigated. A preliminary report on the results obtained is presented herein.

Review of Literature

Attempts to render the bovine udder free from bacteria by infusion with various antiseptics have been in progress since 1887, when Nocard and Mollereau[6] inflated infected quarters with a boric acid solution. Many chemicals have been tried and varying degrees of success reported. In Europe acridine dyes which include entozon, rivanol, uberasan, and trypaflavin have been tried extensively. While as high as 75 per cent success in eliminating *S. agalactiae* from infected quarters treated with trypaflavin has been reported by Steck,[7] Hucker and Lee[8] did not consider acriflavin (English equivalent of the German trypaflavin) as effective, and Schalm[9] found that the use of entozon may cause partial or complete atrophy of treated quarters. Stableforth and Scorgie[10] recommend chemotherapeutic treatment with acriflavin as an adjunct to control by segregation.

The work of Lentz[11] suggested to the writers that silver oxide might be effective in rendering infected quarters of the bovine udder free from mastitis organisms. He found that silver oxide is sufficiently soluble in water to produce a solution which is nontoxic, nonirritating and bactericidal, and that when a nonaqueous dispersion of silver oxide is brought into contact with moist tissues or water, the oxide dissolves as silver hydroxide. The limited amount of solute at saturation was considered to prevent

*From the departments of dairy industry and animal diseases, Storrs Agricultural Experiment Station. Received for publication August 27, 1940.

E. R. Squibb & Sons kindly furnished the colloidal silver oxide (novoxil) used in the experiments described.

[1]Plastridge, W. N., Anderson, E. O., Weirether, F. J., and Johnson, R. E.: Infectious bovine mastitis. Report on a control program based on segregation of infected animals. J. Dairy Sci., xix (1936), pp. 641-650.

[2]Plastridge, W. N.: *Streptococcus agalactiae* as an entity and definite etiological agent in bovine mastitis. Proc. Third Int. Cong. for Microbiol. (1940), pp. 664-665.

[3]Minett, F. C., Stableforth, A. W., and Edwards, S. J.: Studies on bovine mastitis. VIII. The control of chronic streptococcus mastitis. J. Comp. Path. & Ther., xlvi (1933), pp. 131-138.

[4]Stableforth, A. W., Edwards, S. J., and Minett, F. C.: Studies on bovine mastitis. XI. Further observations on the control of chronic streptococcus mastitis. J. Comp. Path. & Therap., xlviii (1935), pp. 200-315.

[5]Muller, J. H.: Ionic silver from silver hydroxide as a therapeutic agent. (Private publication, John Harrison Library of Chemistry, Univ. of Pennsylvania, 1936.)

[6]Nocard and Mollereau: Contagious mammitis of lactating cows. (Title trans.) Ann. de l'Inst. Pasteur, i (1887), pp. 109-126.

[7]Steck, W.: The control of *Streptococcus agalactiae* mastitis. Proc. 12th Int. Vet. Cong., ii (1934), pp. 495-505.

[8]Hucker, G. J., and Lee, D. A.: Mastitis II. The use of certain dyes in the treatment of mastitis. New York State Agr. Exp. Sta. Tech. Bul. 205 (1932).

[9]Schalm, O. W.: The treatment of streptococcic mastitis by infusion of the udder with entozon. J.A.V.M.A., xcvii (1940), pp. 20-27.

[10]Stableforth, A. W., and Scorgie, N. J.: Entozon and acriflavine for the treatment of chronic contagious bovine mastitis. Vet. Rec., l (1938), pp. 663-676.

[11]Lentz, W. J.: Some experiments with a new preparation of silver oxide. Vet. Ext. Quart., Univ. of Pennsylvania, xxxv (1935), pp. 2-15.

effective causticity and at the same time to give a mildly alkaline environment in which normal cell development was possible. According to Lentz, there is no permanent deposit of silver in epidermal cells following application to the skin of animals, and from 75 to 89 per cent of silver applied to the skin as silver oxide is eventually eliminated in the urine and feces. For this reason he believed that practically no ill effects which might result from metallic silver deposition in the body tissue are to be expected.

Material and Methods

The silver oxide used in the experiments described herein was a nonaqueous dispersion of pure oxide of silver in mineral oil. The methods employed in the examination of the milk samples were similar to those previously described in Storrs Agricultural Experiment Station bulletins.[12, 13] Milk samples were examined for macroscopic appearance, reaction to the brom-thymol-blue test, leucocyte count and the presence of mastitis organisms. Streptococci were detected by streaking the surface of a blood-agar plate with a loopful of a 10-cc. portion of the sample which had been incubated at 37° C. for 18 hours. Isolations made from streptococcus-like colonies were identified by biochemic and serologic tests.

Coliform Mastitis

CASE 1 (TURVY TOPAZ STORRS)

A Holstein cow that calved for the first time on July 31, 1938, was found on September 6, 1938, to be infected with coliform organisms in her left hind quarter. During most of her first lactation the milk obtained from this quarter was abnormal in appearance and contained large numbers of leucocytes (6 to 16 million per cc.) and coliform organisms. The gland was atrophic and secreted less than 2 lb. of milk a day during her first lactation. Treatment of the left hind quarter was started August 30, 1939, 13 months after calving.

Procedure.—Samples were collected aseptically prior to milking. All quarters were milked dry, after which 10 cc. of 5 per cent colloidal silver oxide (mineral oil base) was introduced into the milk cistern of the infected quarter with the aid of a blunt-nosed silver cannula* and a hypodermic syringe. This procedure was followed twice a day until six treatments had been given. Following the series of treatments, milking was continued and milk samples for bacteriologic tests were taken once a day prior to the afternoon milking.

Results.—During treatment the treated quarter increased in size until it was about twice that of the opposite quarter. It became hot, hard and tender. This severe inflammation subsided quite rapidly after the last injection. The quarter gradually returned to its original size. During and for two days following the course of treatment the secretion obtained was very thick, gray and odorless. Six days after the last injection the milk secretion was normal in appearance and reacted negatively to the brom-thymol-blue test. After the fourth injection coliform organisms could not be found in the secretion from the treated quarter. After the first injection the leucocyte count increased tremendously (25 to 60 million per cc.); the count was over 8 million while the treatment was in progress and for three days after treatment. Leucocyte counts below a million were obtained 17 days after the last treatment. The treated quarter secreted normal milk, as determined by laboratory tests performed 17 days after the last treatment, and continued to do so until it was dried off.

Because of some difficulty with conception, this animal did not calve again until January 24, 1940. When the udder resumed the functional state prior to calving, it was observed that the left hind (treated) quarter showed uniform turgescence similar to the right hind (normal) quarter. After calving no difference could be observed in size, shape and production between the treated and normal hind quarters. Milk samples taken since calving have all been negative to tests used for detecting evidence of mastitis and remained so until August 1, 1940, the date of the last test. On July 10, 1940, the treated quarter was secreting 17.5 lb. of milk out of a total production of 70 lb.

Streptococcal Mastitis

The University of Connecticut dairy herd has been free of *S. agalactiae* infection for a number of years and could, therefore, not be drawn upon for study. Six animals were selected from the dairy herd of the Mansfield Training School, Mansfield Depot, Conn. All four quarters of each cow were shedding *S. agalactiae*. Three representative cases are reported here. These animals were housed in a barn with 34 other cows. All of the 37 animals were machine-milked; all were infected with *S. agalactiae*. Sanitation and management in this barn were below accepted standards. The animals were held in isolation for slaughter. No spe-

*Special needle No. 6630L, size 2¾", made by Becton, Dickinson & Company, Rutherford, N. J.

[12]Plastridge, W. N., Anderson, E. O., White, G. C., and Rettger, L. F.: Infectious bovine mastitis. 3. Methods of control. Storrs Agr. Exp. Sta. Bul. 197 (1934).

[13]Plastridge, W. N., Anderson, E. O., Williams, L. F., and Weirether, F. J.: Infectious bovine mastitis. 7. Characteristics of udder staphylococci. Storrs Agr. Exp. Sta. Bul. 231 (1939).

cial care was given the three animals on test. It should be mentioned that this farm housed 60 normal animals in another barn.

PROCEDURE

One quarter of each animal was selected for treatment. Selection was based on laboratory findings and clinical evidence. Samples were obtained three times prior to treatment. Clinical examinations were made and the quantity of milk secreted by the selected quarters was determined. Usually milk samples were taken of the fore milk; occasionally stripping samples were obtained.

After a regular afternoon milking each selected quarter was stripped dry and colloidal silver oxide introduced into the milk cistern. After each injection the gland was gently massaged. At the start injections of different concentrations of from 1 per cent to 10 per cent silver oxide were made, but in no instance was a single injection effective in ridding the gland of *S. agalactiae*. One week after the first application daily injections of the test quarters were carried out. These were continued until the milk samples were negative for *S. agalactiae*. Because of the time necessary for making cultural tests, most test quarters received two injections after the milk samples were found to be negative. Before the cows were slaughtered, clinical examinations were made and the quantity of milk secretion from the treated quarters and untreated quarters was determined. The udders were removed immediately after slaughter and examined for gross lesions; tissue blocks were prepared for histologic study. (The gross histopathologic findings will be reported in a later publication.)

CASE 2 (MANSFIELD TRAINING SCHOOL—NO. 50)

This animal, a Holstein cow, gave birth to her fourth calf on May 20, 1939. Her right front and right hind quarters were slack. Laboratory tests were first made during February 1939. All four quarters were shedding *S. agalactiae*. The left front quarter was selected for treatment. The first injection was given November 7, 1939. The left hind quarter was used as a control.

Ten cc. of a 1 per cent dispersion of colloidal silver oxide in a mineral oil base was injected once a day. A total of eight doses was given. The results obtained were as follows:

Clinical Reaction.—When treated once a day (instead of twice a day, as with animal T.T.S.), severe inflammation of the mammary gland did not develop. Some congestion was noticed, but it did not progress to the hot and tender stage.

Macroscopic Appearance of Milk.—Before treatment the milk was normal in appearance. During and shortly after the series of injections, clotty milk was obtained. The milk became normal in appearance four days after the last injection and remained so until the end of the test period (January 11, 1940).

Brom-Thymol-Blue Test. — Before treatment the milk was negative to this test. Positive reactions were obtained during a period of four days after treatment. Thereafter the milk remained normal up to the end of the test period.

Number of Leucocytes.—Before treatment this animal gave milk which contained leucocytes in excess of 1 million per cc. During and immediately after treatment, the leucocyte count increased to 30 to 40 million per cc. Leucocyte counts of 500,000 or less were obtained nine days after the last dose was administered, and continued to be obtained for 18 days. Thereafter leucocyte counts of over 1 million were recorded.

Cultural Tests.—Before treatment all four quarters were shedding *S. agalactiae*. The secretion from the test quarter remained negative for 30 days after treatment (December 18, 1939) after which *S. agalactiae* again appeared in the milk. Two days before the last (eighth) injection the milk did not contain *S. agalactiae*, as indicated by the tests used. During the 30 days when the cultures were negative for *S. agalactiae*, other streptococci regarded as saprophytes were found in the milk samples on five different days.

Combined Laboratory Tests.—Nine days after treatment the milk obtained from the treated quarter was negative to all of the laboratory tests made during a period of twelve days.

Milk Production.—The average afternoon milk production of the treated quarter for a three-day period prior to treatment was 1 lb., 6 oz. The milk production diminished during and immediately following treatment, and then increased gradually. One month after treatment the average production of this quarter was 1 lb., 3 oz. During the same period the production of the left hind quarter dropped from 3 lb., 3 oz. to 3 lb.

Physical Examination.—Before treatment palpation of the test quarter revealed 1+ fibrosis in the dorsal anterior quadrant. Palpation before slaughter on January 12, 1940, showed 1+ fibrosis in the dorsal anterior and ventral posterior quadrants.

CASE 3 (MANSFIELD TRAINING SCHOOL—NO. 55)

This Holstein cow was affected with chronic streptococcal mastitis in all quarters. She delivered her eighth calf on July 8, 1939. The left front and left hind quarters were slack. Laboratory tests were first made in February 1939. The right front quarter was selected for treatment, leaving the right hind quarter as a

control. Treatment was begun November 7, 1939.

Six injections of 10 cc. of a 10 per cent dispersion of colloidal silver oxide in a mineral oil base were given. The results obtained were as follows:

Clinical Reaction.—Same as in animal 50 M.T.S.

Macroscopic Appearance.—Before treatment the milk was thin and clotty. Similar milk was obtained during and shortly after treatment. The milk was normal in appearance five days after the last treatment and remained so for nine days (December 1, 1939); then thin milk was again secreted and continued so until the end of the test period (December 15, 1939).

Brom-Thymol-Blue Test.—Before treatment the milk was suspicious in reaction to this test. A positive reaction was obtained during and up to the fourth day after the last treatment. Negative-reacting milk was then secreted for eleven days, or until November 29, 1939, after which positive reactions were again obtained.

Number of Leucocytes.—Before treatment the leucocyte count varied from 300,000 to 20 million per cc. It was 500,000 or less ten days (November 23, 1939) after the last injection. Low counts were obtained for two days only. Thereafter the count was again high.

Cultural Tests.—Cultural tests were negative for *S. agalactiae* two days before the last injection, and remained so, with the exception of one day, for eleven days or until November 27, 1939.

Combined Laboratory Tests.—At no time during the test period was milk obtained which reacted negatively to all of the tests used.

Milk Production.—Before treatment the average afternoon milk production of the right front quarter was 4 lb., 7 oz. After treatment the average milk yield from this quarter was 3 lb. The untreated quarter produced 3 lb., 13 oz. at the beginning of the test period, as compared with 3 lb., 9 oz. at the end of the period.

Physical Examination.—No clinical examinations were made.

CASE 4 (MANSFIELD TRAINING SCHOOL—No. 36)

This was a Holstein cow which gave birth to her fourth calf on January 4, 1939. All four quarters were secreting milk infected with *S. agalactiae.* The right hind quarter was selected for treatment. The first injection was given November 7, 1939. The remaining three quarters were used as controls.

Ten cc. of a 5 per cent dispersion of colloidal silver oxide in a mineral oil base was injected once a day. A total of six doses was given. The results obtained were as follows:

Clinical Reaction.—Same as in animal 50 M.T.S.

Macroscopic Appearance of Milk.—Before treatment the milk was normal in appearance. During and until the fourth day after the last injection, clotty milk was obtained. Thereafter the milk remained normal in appearance until the end of the test period (January 18, 1940).

Brom-Thymol-Blue Test.—Same results as with animal 50 M.T.S.

Number of Leucocytes.—Before treatment the leucocyte count varied from 500,000 to 2 million per cc. Leucocyte counts of 500,000 or less were obtained 19 days after the last dose was administered, and remained so for 28 days.

Cultural Tests.—Before treatment all four quarters were shedding *S. agalactiae.* With one exception, the secretion of the test quarter remained negative for 37 days after treatment. Two days before the last injection the milk did not contain *S. agalactiae.* During this 37-day period saprophytic streptococci were isolated on six occasions.

Combined Laboratory Tests.—The milk obtained from the treated quarter was negative to all of the laboratory tests used for 15 consecutive days.

Milk Production.—The test quarter produced on the average 2 lb., 9 oz. of milk before, and 2 lb. of milk after treatment. The combined production of the other three quarters was 6 lb., 6 oz. at the beginning of the test period, as compared with 5 lb., 1 oz. at the end of the period.

Physical Examination.—Before treatment palpation of the test quarter revealed 2+ fibrosis in the dorsal posterior quadrant and on the border between the dorsal and ventral quadrants; in the midline there seemed to be a small chestnut-size lump. At the end of the test period palpation revealed 2+ fibrosis in the dorsal and ventral posterior and the anterior ventral quadrants. The chestnut-size lump was still present. This animal has not been slaughtered (August 1, 1940).

Comment

Preliminary observations on the influence of colloidal silver oxide on three quarters infected with *Streptococcus agalactiae* suggest that the milk from the treated quarters was rendered free from the common mastitis organism for periods of from 11 to 37 days. Under the conditions of the experiment the reappearance of *S. agalactiae* in the milk may have been caused by reinfection from without or from organisms which were located in remote lesions within the gland and had escaped the bactericidal action of the silver oxide. One quarter

Effect of Novoxil Liquid on Catarrhal (Chronic) Mastitis and on Streptococcus Agalactiae Udder Infection*

LOUIS A. KLEIN, V.M.D., Sc.D., ALBERT L. KLECKNER, Ph.D., and ROBERT O. BILTZ, V.M.D.

Philadelphia, Pa.

THE INVESTIGATION which is the subject of this report was made to determine the effect of novoxil liquid, when injected into the milk cistern, on catarrhal (chronic) mastitis of the cow; on *Streptococcus agalactiae*, the essential cause of most cases of this type of bovine mastitis; and on the udder tissues and milk secretion. Novoxil liquid† is a colloidal dispersion of silver oxide in a light mineral oil.

EXPERIMENTAL COWS

Twenty-three cows with a natural infection of *S. agalactiae* were used in our experiments. Clinical symptoms of catarrhal (chronic) mastitis were present in ten of these animals. Five different breeds were represented in the group: Ayrshire, Brown Swiss, Guernsey, Holstein and Jersey. Eighteen of the cows came from four different herds and throughout the experiments occupied a stable on one of the University farms on which there were no other cows. They were milked twice daily and otherwise cared for by one man, an experienced dairyman, who had no contact with any other cattle. The cows were placed in the stable as they were acquired and were removed and disposed of as our work with them was completed, so that they were not all in the stable at any one time. Before milking each cow, the attendant washed his hands with soap and water, rinsed them in an antiseptic solution and wiped them dry. At each milking, the first few streams from each quarter were drawn into a strip cup with a black cloth strainer and examined for flakes and other abnormalities. A record was kept of the milk production while the cows were in the stable. In selecting the cows, preference was given to those showing clinical symptoms of mastitis and having the most complete history. The information desired included the age, period of lactation, stage of lactation, calving record, date of last breeding, and mastitis record. Five of the 23 cows used in the investigation remained on the owner's farm in stables with the other cows in the herd but were always milked by the same man before he milked any of the other cows. These five cows were the only cows in the herd infected with *S. agalactiae* infection as indicated by the results of repeated bacteriologic examinations.

PLAN OF EXPERIMENTS

Before the novoxil liquid was injected, a physical examination of the udder was made and separate samples of milk from each quarter were examined by the usual bacteriologic methods for *S. agalactiae*, precautions being taken against external contamination. The examination of the milk was repeated four or more times on each cow to insure the detection of all infected quarters.

The novoxil liquid was injected through the

*From the School of Veterinary Medicine, University of Pennsylvania. Received for publication March 27, 1941. This investigation was supported by the Bureau of Animal Industry of the U. S. Department of Agriculture, Pennsylvania Department of Agriculture, Pennsylvania Department of Health, E. R. Squibb & Sons, and the University of Pennsylvania, the latter being assisted by contributions from herd owners and milk distributors.

†Supplied by E. R. Squibb & Sons.

(*Continued from preceding page*)

which was affected with coliform mastitis before and for a period of twelve months following the first calving received two injections daily for six days and became completely normal following the subsequent calving. Injection of colloidal silver oxide caused a mild irritation of the udder when given once a day and a marked irritation when given twice daily. However, none of the treated quarters appeared to be permanently injured.

While the results presented are based on preliminary observations, they appear to be sufficiently promising to justify further investigation of colloidal silver oxide as an aid in the control of bovine mastitis.

teat canal into the milk cistern of infected quarters immediately after the udder had been milked out at a regular milking. Before the injection was made, the udder and flanks were washed with an antiseptic solution and then dried, individual paper towels being used for the latter purpose. The ends of the teats were wiped with cotton saturated with 70 per cent alcohol, the two left teats first.

The novoxil liquid was supplied in bottles containing 100 cc. Before a bottle was taken to the stable, it was vigorously shaken to obtain an equal distribution of the silver oxide in the oil, and in cold weather, was carried to the stable in an inside pocket to keep the liquid warm.

Sterile 10-cc. glass syringes and blunt cannulas were used in making the injections. The cannulas are 14-gauge, approximately 2⅜ in. long, and are bent at a right angle 1¼ in. from the end to which the syringe is attached. Immediately before the syringe was filled, the bottle was again shaken thoroughly and the stopper was wiped with alcohol. A cannula was then inserted and allowed to remain in position until all the liquid required was withdrawn, another cannula being attached to the syringe to make the injection. Infected quarters on the right side were injected first.

At a regular milking time 12 or 24 hours after each injection, the udder was examined for evidence of irritation and at the same time a separate sample was taken of the first milk from each quarter, after the first few streams were discarded, the usual precautions being taken to avoid contamination. The macroscopic appearance of each sample was noted and it was then examined bacteriologically. These examinations were repeated after each injection. The streptococci usually failed to develop on the ox-blood-agar plates after the first injection, but as some of the silver injected was being eliminated in the milk samples and as it was possible that this might inhibit the growth of the organism in the plates, the examination of the milk of each quarter injected was continued for three weeks after the last injection, unless the infection reappeared. Cows under treatment or under observation following treatment were always milked before the other cows in the stable. At the end of the period of observation, several of the cows were slaughtered and the udder examined macroscopically.

PRELIMINARY TRIALS

Preliminary tests to determine the germicidal effect of novoxil liquid on *S. agalactiae* and its effect on the udder were made on seven cows with 20 infected quarters, nine of which showed clinical symptoms of catarrhal (chronic) mastitis. Preparations of novoxil liquid containing 2.5, 3, 5 and 10 per cent silver oxide were injected in different udder quarters. Five to 20 cc. of the same concentration was injected one to ten times at intervals of 12, 24, 48 and 72 hours. At first only a single quarter was injected, then two on the same side of the udder, and finally all four quarters.

The results of this preliminary work are summarized in table I. The *S. agalactiae* was destroyed in all but one of the 20 infected quarters, and the symptoms of mastitis disappeared in two to seven days from six of the nine quarters so affected. The one quarter in which the infection survived, which was also one of the three in which the symptoms persisted, received only a single injection of 20 cc. of the 5 per cent concentration. Evidence of the presence of the organism was not observed on the blood-agar plates for seven days following the injection.

A marked change was observed in the appearance of the milk in all cases. At the first milking following the first injection, it was thick and gray. After the last injection it became less thick and lighter in color with each succeeding milking and was normal in appearance by the second to the tenth day; in over one half of the quarters by the fifth day, except in three quarters affected with mastitis.

The udder invariably showed a marked reaction. When examined 12 to 24 hours after the first injection, the injected quarters were swollen and firm and there was a subcutaneous edema along the ventral border. In some instances, the swelling was warm and sensitive, and there was a rise in the body temperature. These changes continued in about the same degree when the injections were repeated at intervals of 12, 24 or 48 hours, but when the intervals were extended to 72 hours, there was some retrogression between the injections. The intensity of the reaction corresponded closely to the concentration and the quantity introduced into the quarter. The udder returned to the pre-treatment condition in from 4 to 12 days, generally in about 7 days. The injection of 20 cc. of the 5 per cent concentration caused a pronounced reaction in the udder tissues and this quan-

TABLE I—Novoxil Liquid Injections: Preliminary Trials

Cow No.	Quarters Treated	Quantity Novoxil Injected (cc.)	Conc. Silver Oxide (%)	No. of Injections	Hours Between Injections	Appearance of Milk Before Treatment	Days Required for Milk to Return to Normal	Days Required for Udder to Return to Normal*	Examination for S. Agalactiae After Treatment
S-1	LH	10	2 5	10	12	Flakes	2	10	Negative
S-3	LH, LF	10	2 5	10	12	Yellow clots	7	8	Negative
S-4	LH, RH	10	3 0	4	72	Normal	4	5	Negative
S-5	LF	10	5 0	2	72	Normal	5	5	Negative
S-8	RH, RF	10	5 0	4	72	Normal	5	6	Negative
S-5	RF	10	5 0	5	24	Normal	10	12	Negative
S-8	LH, LF	10	5 0	5	24	Normal	4	6	Negative
S-10	LH, LF	10	5 0	5	24	Cream colored	5	8	Negative
S-5	RH	20	5 0	1		Flakes, cream colored	Flakes, cream colored		Negative 7 days only
S-2	RH	20	5 0	5	24	Clots	Watery, cream colored	16	Negative
S-3	RH, RF	5	10 0	4	48	Normal	7	7	Negative
S-1	RH	10	10 0	4	72	Normal	4	4	Negative
S-10	RH, RF	10	10 0	4	72	Flakes	RH—cream colored 5 (RF)	12	Negative

TABLE II—Novoxil Liquid Injections: 5 Per Cent Silver Oxide, 10 cc. Each Injection

Cow No.	Quarters Treated	No. of Injections	Hours Between Injections	Appearance of Milk Before Treatment	Days Required for Milk to Return to Normal	Days Required for Udder to Return to Normal*	Examination for S. Agalactiae After Treatment
S-13	RH	2	72	Cream colored, few clots	4	6	Negative
S-16	All	2	72	Normal	7	7	All quarters infected
S-17	RF	2	72	Normal	7	7	Negative
S-18	All	2	72	RF—cream colored	RF—cream colored	6	RH negative, others infected
S-19	LH	2	72	Normal	14	14	Negative
S-20	LF, RH, RF	2	72	Normal	11	11	RH, RF negative, LF infected
S-23	LF, RH, RF	2	72	Normal	4	12	Negative
S-24	RF	2	72	Normal	4	8	Negative
S-6	All	4	72	LH—clots; RH—clots, cream colored	4 (LF, RF) LH, RH—cream colored, nearly dry	9	LF, RH, RF negative, LH infected
S-7	All	4	48	LF, RF flakes	5	7	Negative
S-12	All	2	24	Normal	3	6	All quarters infected
S-11	All	3	24	Normal	4	12	Negative
S-14	All	4	24	Normal	6	11	Negative
S-9	All	5	24	Clots and watery	LH—clots, cream colored; others nearly dry	17	Negative

TABLE III—Repeat Treatments of Cows Failing to Respond to First Treatment

Cow No.	Quarters Treated	Quantity Novoxil Injected (cc.)	Conc. Silver Oxide (%)	No. of Injections	Hours Between Injections	Appearance of Milk Before Treatment	Days Required for Milk to Return to Normal	Days Required for Udder to Return to Normal*	Examination for S. Agalactiae After Treatment
S-5	RH	10	5 0	5	24	Flakes and cream colored	10	12	Negative
S-12	All	10	5 0	2	72	RH—cream colored, others normal	3	6	Infected
S-12	All	10	2 5	3	72	Normal	3	3	Infected
S-16	All	10	3 0	3	72	RF—flakes	6	2	RF—negative, others infected
S-18	LH, LF, RF	10	3 0	3	72	RF—cream colored, flakes	LF, RF—cream col. and clots	2	Infected
S-18	LH, LF, RF	10	3 0	4	24	LF, RF—cream colored and clots	No change	2	Infected
S-18	LH, LF, RF	10	5 0	4	24	LF, RF—cream colored and clots	No change	1	Infected
S-18	LH, LF, RF	10	5 0	1†	0	LF, RF—cream colored	LH, LF—cream colored, RF brown	1	Infected
S-25	LF	10	5 0	1†	0	Normal	2	2	Negative

*Normal is used to indicate that the udder returned to the pre-treatment condition.

†Novoxil liquid injected after the afternoon milking, and the udder was not milked out until the following day at the regular time of the afternoon milking.

tity was used on only two quarters. In one of these cows (S-5) the udder failed to return to normal in 30 days. (See table III.) The udder of the other cow returned to normal in 16 days.

TREATMENT EXPERIMENTS

Following these experiments, 14 cows were given two to five 10-cc. injections of the 5 per cent concentration at intervals of 72, 48 or 24 hours. The data obtained from these experiments are presented in table II.

Eight of the 14 cows with 18 quarters infected were given two 10-cc. injections 72 hours apart. One quarter in each of two of these cows (S-13, S-18) exhibited clinical symptoms of catarrhal (chronic) mastitis. S-18 had all four quarters infected with *S. agalactiae*. The symptoms disappeared from the affected quarter in S-13 but not from the affected quarter in S-18.

Five of these eight cows, including S-13, became entirely free of the infection, and the infection was destroyed in three of the eleven infected quarters in the three other cows, including one quarter in S-18. The milk from the treated quarters showed the same changes following the injections as were observed in the experimental tests but returned to normal in from 4 to 14 days after the last injection, except in the one quarter affected with mastitis in S-18. The swelling following the injections disappeared in 6 to 14 days.

Two of the 14 cows (S-6, S-7) with eight quarters infected, two in each cow showing clinical symptoms of catarrhal (chronic) mastitis, were given four injections at 72- and 48-hour intervals, respectively. The symptoms of mastitis disappeared from the two affected quarters in S-7 and the infection was destroyed in all four quarters, but the mastitis symptoms persisted in both affected quarters of S-6 although the infection was destroyed in one of these quarters and two others. The secretion of quarters treated showed the usual changes but became normal in appearance in four and five days, respectively, except in the two affected quarters of S-6. The swelling caused by the injections disappeared from the udders in nine and seven days, respectively.

The last four cows listed in table II, with 16 infected quarters, all four quarters in one cow (S-9) showing symptoms of catarrhal (chronic) mastitis, were given two, three, four and five injections, respectively, at 24-hour intervals. The infection persisted in the cow receiving two injections but was entirely destroyed in the two cows receiving three and four injections, respectively, each having had all four quarters infected. The other cow in this group (S-9) also became free of the infection but the symptoms of mastitis persisted. This cow received five injections. Milk from the treated quarters showed the usual changes but was again normal in three to six days following the last injection, except in the cow (S-9) showing clinical symptoms of mastitis. The swelling following the injections disappeared from the udder in from six to twelve days after the last injection in the cows which received two to four injections, but in the cow (S-9) which received the five injections it took 17 days for the swelling to recede from the udder. It does not appear that age, stage of lactation, previous attacks of mastitis or extent of induration of the udder had any significant influence on the results of treatment in this group of cows or in those in the preliminary trials.

REPEATED TREATMENTS

Three of the cows (S-12, S-16 and S-18) that failed to respond to two injections at intervals of 24 or 72 hours (table II), one cow (S-5) that failed to respond in one quarter to a 20-cc. injection (table I), and one cow (S-25) which was injected without result when "dry" (table IV), were subjected to additional treatments. Referring to table III, it will be seen that S-12 resisted a second and third treatment, and S-18 four additional treatments. On the other hand, a second treatment freed both S-5 and S-25 of the infection, which was present in one quarter of each cow, while a second treatment of S-16 resulted in the infection being destroyed in one of four infected quarters. It should be noted that novoxil liquid of

TABLE IV—Treatment of "Dry" Cows: One Injection, 10 cc. Novoxil Liquid, 5 Per Cent Concentration

Cow No.	Quarters Treated	Condition of Udder	Days Before Calving	Appearance of Milk After Calving	Condition of Udder After Calving	Examination for S. Agalactiae When Fresh
S-15	LH, LF, RF	"Springing"	10	Normal	Normal	Negative
S-12*	All	"Springing"	8	Normal	Normal	Negative
S-25	LF, RF	"Springing"	9	Normal	Normal	RF negative, LF infected

*This cow was treated previously on three different occasions in the preceding lactation period. (See tables II and III.)

less than 5 per cent concentration was used in four of the treatments. There was no apparent injurious effect from the repeated treatments. The last treatment of S-18 and the treatment of S-25 differed from that used on any of the other cows in this investigation, a single injection of 10 cc. of novoxil liquid (5 per cent concentration) being made into the infected quarters immediately after an afternoon milking and allowed to remain until the next afternoon milking. The reaction of the udder was no greater than in the cows which were milked about twelve hours following injection.

Treatment of "Dry" Cows

During the course of this work, three cows were treated with novoxil liquid during the "dry" period. All of these cows had a history of *S. agalactiae* infection in the previous lactation period; two of the cows had a history of infection for two or more lactation periods. *S. agalactiae* was isolated in all instances from the secretion the last time the quarters were milked out at the end of the "drying off" period.

The results of this method of treatment are presented in table IV. The quarters in which the infection was present when the cows were "dried off" were injected once with 10 cc. of the 5 per cent concentration at the time when the udders were beginning to show evidence of increased activity ("springing" stage). The quarters were not milked out after the injections were made. The injections were made ten, eight and nine days, respectively, before the cow calved. In no case was there any evidence of irritation or any unusual swelling in the quarters either before or after calving. The secretion had a normal appearance from the very beginning of lactation.

As is shown in table IV, the *S. agalactiae* infection was entirely destroyed in two cows, and in one of the two infected quarters in the other (S-25). This cow, according to the history, had a more recent infection than the others. The current "freshening" was its second and the infection developed some time during the previous or first lactation period. This cow was "dried off" early and had a "dry" period of about six months.

Another point of interest in these experiments was the successful result in the case of cow S-12. This animal, infected with the beta-hemolytic variety of *S. agalactiae* in all four quarters, had been treated on three different occasions during the last three months of the previous lactation period without the infection being destroyed. (See tables II and III.) The known history of *S. agalactiae* infection in this cow extends back over nearly two complete lactations, covering a period of approximately two years.

Influence of Treatment on Milk Production

During the period of treatment there was a reduction in the quantity of milk produced ranging from 4 to 53 per cent, but in one half of the cows treated there was a decrease of only 4 to 25 per cent. After the last injection, there was a gradual rise in the quantity of milk produced and by the end of the period of observation, usually three weeks after the last injection, the

TABLE V—Spectrochemical Examinations for Silver

Cow No.	Quarters Treated	Total Quantity Novoxil Injected (cc.)	Conc. Silver Oxide (%)	Number of Injections	Total Quantity Silver Oxide Injected per Quarter (Gm.)	Days Required for the Elimination of the Silver
S-5	RH, RF	50	10	5	5.0	73 to 106
S-8	LH, LF	50	5	5	2.5	88 to 121
S-3	LH, LF	100	2.5	10	2.5	80 to 113
S-14	All	40	5	4	2.0	36 to 52
S-7	All	40	5	4	2.0	68 to 101
S-3	RH, RF	40	5	4	2.0	62 to 95
S-11	All	30	5	3	1.5	36 to 52
S-4	All	40	3	4	1.2	82 to 99
S-13	RH	20	5	2	1.0	36 to 52

production amounted to 62 to 92 per cent of the pre-treatment quantity.

Effect on the Udder

Treated quarters returned to their pre-treatment condition in 5 to 13 days, as determined by physical examination. Indurations were not affected.

A macroscopic examination of eleven of the udders was made *post mortem*. On section, the glandular portion of the treated quarters was found to be gray or bluish-gray. In some section surfaces, the color extended from the milk cistern to the dorsal border of the quarter; in others up to the middle third, indicating the extent of the diffusion of the liquid injected.

Elimination of Silver

The greater quantity of silver injected into a quarter was eliminated in about five days following the last injection, as indicated by the appearance of the secretion.

The milk from 25 treated quarters in eight cows was examined spectrochemically at various intervals following the last injection. The samples for this examination were taken from the total quantity of milk obtained from the treated quarters at a regular milking.

The first samples were taken from the nine treated quarters of three of the cows (S-11, S-13, S-14) five days after the last injection. The quantity of silver found in these samples was 1.6, 1.0 and 2.5 parts per million, respectively. Samples collected from these cows on the 36th day after the last injection contained 0.006, 0.007 and 0.01 p.p.m., respectively, while samples collected on the 52nd day were negative. Table V shows the time required for the silver to disappear from the milk from the 16 treated quarters of the five other cows, the shortest period being from 62 to 95 and the longest from 88 to 121 days after the last injection. The last column of the table gives the data for each cow, the first figure in each case representing the time of the last positive examination and the second figure the time of the next succeeding examination, which was negative. The quantity of silver present in the last positive examinations ranged from 0.005 to 0.05 p.p.m. In this group of cows, there does not appear to be any direct correlation between the time required for the silver to be entirely eliminated and the quantity injected, as the data in table V show.

Summary and Conclusions

Twenty-three cows with 67 quarters infected with *Streptococcus agalactiae* received injections of novoxil liquid in the infected quarters. Ten of the cows showed clinical symptoms of catarrhal (chronic) mastitis in 19 of the infected quarters. Novoxil liquid is a colloidal dispersion of silver oxide in a light mineral oil.

Novoxil liquid containing 2.5, 3, 5 and 10 per cent silver oxide was injected into different udder quarters. Five to 20 cc. of the same concentration was injected one to ten times at intervals of 12, 24, 48 and 72 hours. All but eight of the cows received a single course of treatment while in milk. Five were given repeated courses of treat-

ment while in milk and three received a single injection during the "dry" period.

The *S. agalactiae* infection was destroyed in 59, or 88.1 per cent, of the infected quarters. The infection was destroyed in all infected quarters in 19, or 82.7 per cent, of the cows treated, thus freeing them of the infection entirely.

Ten, or 52.6 per cent, of the quarters showing clinical symptoms of catarrhal mastitis recovered from the symptoms and the infection was destroyed. The clinical symptoms of mastitis and also the infection disappeared from all of the quarters in five, or 50 per cent, of the cows showing clinical symptoms of catarrhal (chronic) mastitis before treatment.

The results were satisfactory in the few cows treated with the 2.5 and 3 per cent concentration, but the reaction of the udder was not much greater with the 5 per cent concentration and the latter was used in most cases as it was assumed that it would be more certain in its effects.

Injections at 24-hour intervals appear to be more effective than at 72-hour intervals but only approximately one third of the cows treated received injections at 24-hour intervals. Three to five injections were more effective than a lesser number. These statements should not be regarded as conclusive, however, because of the limited number of cows involved.

Swelling of the udder and a change in the appearance of the secretion occurred in all cases following the injections, but these changes were only temporary.

The greater quantity of the silver injected into a quarter was eliminated in about five days following the last injection, but traces were present in the milk for some weeks later.

Studies on Control of the Liver Fluke of Cattle in the Hawaiian Islands*

JOSEPH E. ALICATA, Ph.D.
Honolulu, T. H.

LIVER-FLUKE infection represents one of the major diseases of beef and dairy cattle in the Hawaiian Islands. According to available information, there are at present about 130,000 beef and 8,700 dairy cattle on the various larger islands of the Hawaiian group, including Niihau, Kauai, Oahu, Molokai, Lanai, Maui and Hawaii. Of these, it has been roughly estimated that 50 per cent of the beef and 80 per cent of the dairy cattle are infected with flukes. In one group of 419 dairy cows from the Island of Oahu, slaughtered at the Hawaii Meat Company in Honolulu during 1939, 385, or 91.8 per cent, showed flukes or fluky lesions in the liver.

In 1892, Lutz[1] first reported this infection common in four of the larger islands. Although Lutz reported the local liver fluke as *Fasciola hepatica*, a more recent study by Alicata and Swanson[2] has shown the parasite to be *Fasciola gigantica*.

FACTORS INVOLVED IN THE MAINTENANCE AND SPREAD OF FASCIOLIASIS IN HAWAII

The maintenance of fluke infection in Hawaii, and undoubtedly elsewhere, is largely dependent on various factors of which topography, climatic conditions and agricultural practices are most important.

The islands of the Hawaiian group represent the summits of a 2,000-mile range of volcanic mountains. These mountains are of comparatively recent origin and steep, and vary from coastal to centric or eccentric in position. They descend to the ocean abruptly, in steep walls, or by gradual transition over relatively flat land with very little drainage. These poorly drained lowlands and valleys, especialy on the windward side, often represent rather extensive swamps. Rainfall is most prevalent in winter months, but showers during other seasons of the year maintain the swampy conditions.

Although the Hawaiian Islands lie within 19° and 23° north latitude and within the "tropical zone," they enjoy a mild and uniform climate, chiefly because of their position in the midst of a large body of water and the presence of mountains in the path of the northeast trade winds. The average temperature is about 70° to 72° F., and the mean temperature varies only about 5 to 8 degrees from the coldest to the warmest months. The poorly drained lowlands and the mild climate make this region suitable for snail propagation the year around as well as for the development and hatching of fluke eggs, and increase the longevity of fluke cysts on vegetation.

Moreover, local agricultural practices have not hindered the maintenance of infection. With ample supplies of vegetation, cattle are allowed to graze continually. Also, many of the dairymen have for a long time been in the habit of feeding to cattle cut forage from wet and swampy areas. These practices are largely responsible for the widespread fluke infection in the Territory.

METHODS OF FLUKE CONTROL

The methods of fluke control which have received attention and are being advocated in Hawaii are similar to those practiced elsewhere. They involve (1) control of the fresh-water snail *(Fossaria ollula)*, which serves as an intermediate host; (2) protec-

*Technical paper No. 71 of the Hawaii Agricultural Experiment Station. Presented before the Section on Sanitary Science and Food Hygiene at the 77th annual meeting of the American Veterinary Medical Association, Washington, D. C., August 26-30, 1940.

[1]Lutz, A.: Zur Lebensgeschichte des *Distoma hepaticum*. Cent. f. Bakt. u. Paras., xi (1892), pp. 783-796.

[2]Alicata, J. E., and Swanson, L. E.: *Fasciola gigantica*, a liver fluke of cattle in Hawaii, and the snail *Fossaria ollula*, its intermediate host. J. Parasitol., xxiii (1937), pp. 106-107.

tion of the animals from infection; and (3) destruction of the flukes in cattle by the use of anthelmintics.

Snail Control.—Two methods of snail control have been studied, namely, the broadcasting of copper sulfate in snail-infected streams or swamps, and biological control. Copper sulfate is one of the best known chemicals for the destruction of snails. Dr. L. E. Swanson of the U. S. Bureau of Animal Industry demonstrated that this method was effective in Hawaii. He found that, owing to the quantity of vegetation in local streams and swamps, a concentration of 1 part of copper sulfate to 300,000 parts of water was in many cases more effective in destroying snails than the more common concentration of 1 to 500,000 parts.

The biological control of snails has received consideration in the past few years from the writer. One of the first snail predators studied under experimental conditions was the crayfish *(Astacus nigrescenes)*, introduced into the Territory a few years ago and now common in taro patches on the windward side of Oahu. Several hundred fresh-water snails, including *F. ollula*, were placed in a shallow, artificially constructed pond about 20′ x 4′ in size, containing vegetation and about 20 crayfish. All of the snails were devoured by the crayfish during the course of a few days. In all instances the crayfish cracked the shells in order to get to and devour the snails. Investigation into the habits of the crayfish in taro patches showed that they were making holes in the banks of the patches and occasionally destroying the taro corms. It was, therefore, believed unwise to release the crayfish in the swamps.

The possibility of utilizing fireflies as an instrument of biological control procedure also has been considered. Although this method has not been utilized in liver-fluke control, the *Journal of the New York Entomological Society*[3] and Okada[4] (1928) reported that Japanese fireflies *(Luciola lateralis and L. cruciata)* have been utilized in Japan in the control of certain snail carriers of the human blood flukes. In correspondence with Dr. Hochiro Yuasa, formerly entomologist of the Kyoto Imperial University, the writer was informed that these two Japanese aquatic fireflies are exclusively carnivorous. The larvae of *L. lateralis* live in muddy standing water and feed on fresh-water snails. Because these insects are regarded as beneficial, they are protected by law in certain districts of Japan.

As a result of this information, Mr. D. T. Fullaway and Mr. Noel Krauss of the Territorial Board of Commissioners of Agriculture and Forestry, while in Japan during 1939, collected and shipped to Hawaii a quantity of adults of the two Japanese fireflies, which were liberated on Oahu. In addition, Mr. Fullaway, while visiting the Philippines, collected and shipped to Hawaii four species of fireflies (Luciola spp.). These insects, as well as additional eggs which are received periodically by Mr. Fullaway, are being reared. The larvae are carnivorous and devour various fresh-water snails, including those which transmit liver flukes. The larval stage is said to last from about one to two months. As soon as these insects reach the adult stage, they are being liberated in suitable locations. At the present time, hundreds of adults have been freed and it is hoped that they will become established.

Protection of Cattle from Infection.—This method of control involves the common procedure of fencing off infested areas, preventing grazing in swamps, and avoiding vegetation cut from possibly infested areas.

In 1936 and 1938, the writer[5, 6] suggested the possible value of silage in fluke control, based on the observation that fluke cysts were destroyed in three-month-old silage made from honohono and panicum. This possibility requires additional investi-

[3] J. New York Ent. Soc., xxxv (1927), p. 315.

[4] Okada, Y. K.: Two Japanese aquatic glow-worms. Trans. Ent. Soc. London, lxxvi (1928), pp. 101-108.

[5] Alicata, J. E.: Silage as a possible means of killing liver fluke larvae encysted on grass. Hawaii Agr. Exp. Sta. Rpt. (1936), p. 87.

[6] *Ibid.*: Observations on the life history of *Fasciola gigantica*, the common liver fluke of cattle in Hawaii, and the intermediate host, *Fossaria ollula*. Hawaii Agr. Exp. Sta. Bul. 80 (1938), 22 pp.

gation; it may be a desirable approach, especially in certain parts of the continental United States where silage is more widely used. Ensilage would permit the utilization of vegetation from wet areas which would otherwise be unsafe.

Treatment of Cattle for Flukes.—Treatment for flukes not only improves the general health of the cattle (figs. 1, 2, 3), but also reduces pasture infection.

Among the more important flukecidal drugs are hexachloroethane, kamala and a proprietary drug known as distol. According to information on file at this station, obtained from the late Dr. M. C. Hall, Hilz and Schauble found hexachloroethane 100 per cent effective when given in doses of 20 to 30 Gm. for each 50 kg. of body weight. De Blieck and Baudet also found it effective and recommended a dose of 10 Gm. for each 25 kg. of body weight, distributed over a period of four days. According to Hutyra and Marek,[7] distol has been used by Marek, Reisinger and others and has been found highly effective in the treatment of cattle for flukes. Each capsule of distol, according to the label, is composed of 10 Gm. of hexachloroethane, 1 Gm. of aspidinofilic acid (conc.), and 1.6 Gm. of talc.

Some of the early studies in this station on treatment with distol, conducted by Swanson and Goo,[8] and with kamala, by Goo and Alicata[9] were reported in the annual reports of this station for 1937 and 1938, respectively. Further details of these studies were reported by Alicata, Swanson and Goo[10] in 1940. In the past two years, studies have been conducted by the writer to obtain more evidence on the efficacy of these and other drugs and to determine the least amount of drugs necessary to destroy infections of various degrees of severity. The degree of fluke infection has been determined by fluke-egg count before treatment. After treatment, 20 days were allowed to elapse before the feces were reëxamined for fluke infection. An explanation of the egg-count method will be given later in this paper. The results of administration of various drugs to fluke-infected cattle are summarized below.

1) TREATMENT WITH DISTOL

a) *Dosage: One capsule to 70 lb. of body weight, administered in two days.*

Forty-one animals were treated with distol at the rate of one capsule to 70 lb. of body weight, given on two successive mornings, with about twelve hours of starvation before treatment and three hours after each treatment. These animals ranged in weight from 320 to 977 lb., and the total number of capsules administered to each ranged from 5 to 14.

The results, which are given in table I, indicate that in most cases of infection ranging from one to 45 eggs per gram of feces, the drug destroyed all of the flukes; it is, however, impossible to determine, in a live animal, whether or not young and non-egg-laying flukes are destroyed. In heavier cases of infection (more than 50 eggs per gram of feces) the drug did not usually destroy all the flukes, but reduced the egg count to a considerable degree.

b) *Dosage: One capsule to 70 lb. of body weight, administered in three days.*

An additional 16 animals, with egg counts ranging from one to 40 per gram of feces, and weighing from 400 to 817 lb., were treated at the rate of 1:70 administered over three successive days. In all cases no eggs were recovered from the feces following treatment. Another animal, in extremely poor condition, with a fluke-egg count of about 1,255, was treated at this rate and showed a count of 9.8 eggs per gram of feces after treatment. This animal eliminated a large number of dead flukes with the feces during the second day of the treatment.

c) *Dosage: One capsule to 80 lb. of body weight, administered in two days.*

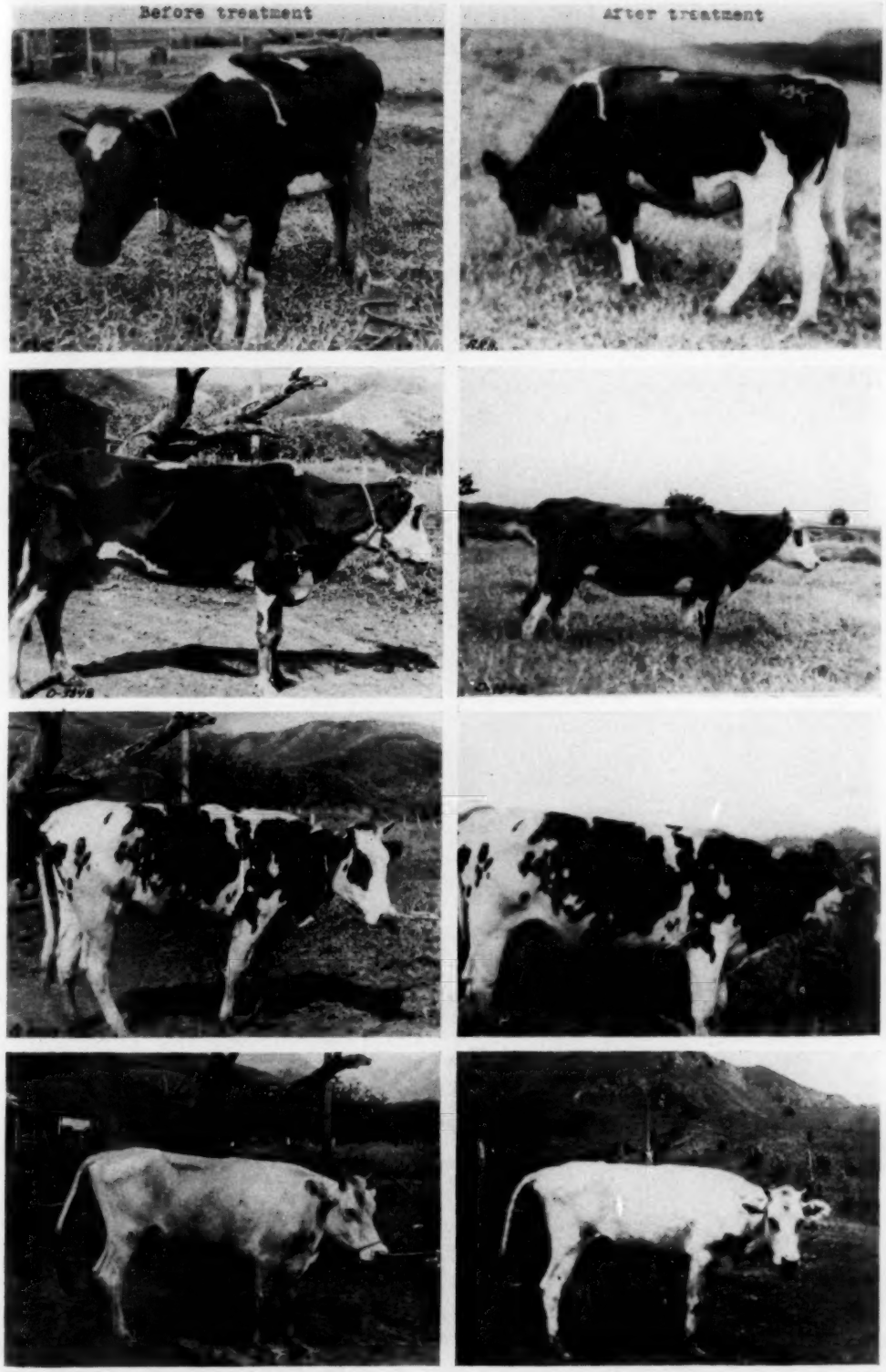
Twenty-one animals were treated at the rate of 1:80 in order to determine whether or not this lower dosage would be satisfactory in treating light infection. These animals ranged from 391 to 1,250 lb. in weight and the number of capsules administered to each animal varied from five to six; the fluke-egg counts ranged from one to 19. The results showed that 17 of

[7]Hutyra, F., and Marek, J.: Special Pathology and Therapeutics of the Diseases of Domestic Animals (Alexander Eger, Chicago, 1926), ii, 850 pp.

[8]Swanson, L. E., and Goo, G. W. H.: Liver fluke control—drug experiments. Hawaii Agr. Exp. Sta. Rpt., 1937 (1938), pp. 90-93.

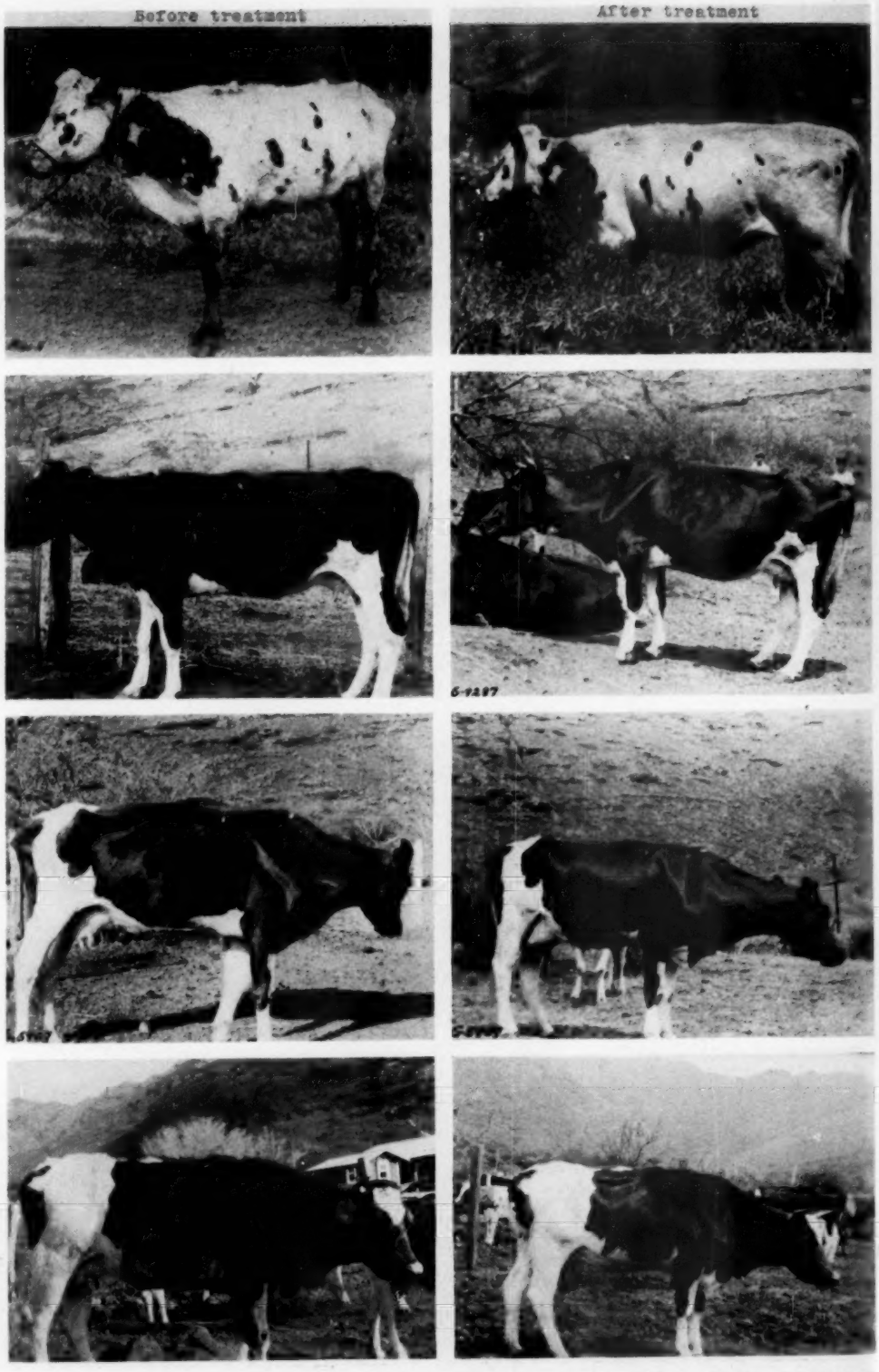
[9]Goo, G. W. H., and Alicata, J. E.: Treatment of fluke-infested cattle with kamala. Hawaii Agr. Exp. Sta. Rpt., 1938 (1939), p. 78.

[10]Alicata, J. E., Swanson, L. E., and Goo, G. W. H.: Methods of controlling liver flukes of cattle in Hawaii. Hawaii Agr. Exp. Sta. Circ. 15 (1940), 23 pp.



—After Alicata, Swanson and Goo, 1940.

Fig. 1. Recovery from fascioliasis. Left: Cows at the time of treatment with distol. Right: Same cows four months later.



—*After Alicata, Swanson and Goo, 1940.*

Fig. 2. Recovery from fascioliasis. Left: Cows at the time of treatment with kamala. Right: Same cows four months later.

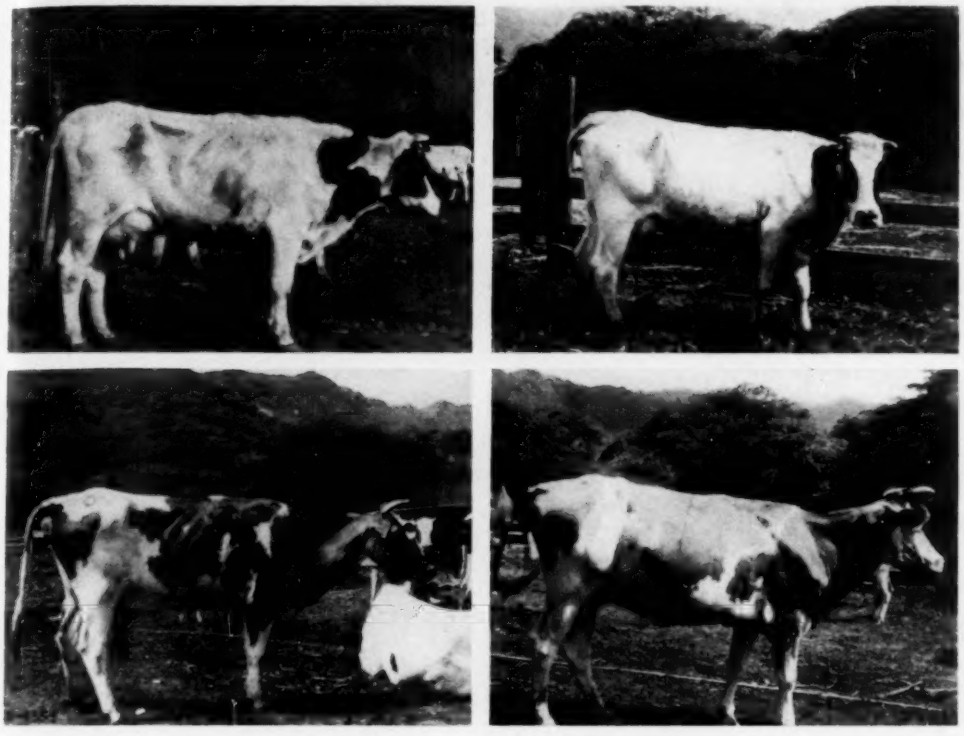


Fig. 3. Recovery from fascioliasis. Left: Cows at the time of treatment with hexachloroethane combined with kamala extract. Right: Same cows four months later.

these animals were negative for fluke infection and four remained positive.

2) TREATMENT WITH KAMALA

a) *Dosage: One capsule to 60 lb. of body weight, administered in four days.*

Thirty dairy cows were treated at the above dosage, each capsule containing 10 Gm. of kamala. The weight of the animals ranged from 690 to 1,241 lb., and the total drug administered to each ranged from 11 to 21 capsules. The results, which are summarized in Table II, show that in all cases the animals were negative after treatment. The drug, however, appeared to have a toxic effect as most of the animals developed exhaustive diarrhea which lasted from one to two weeks. One of the animals, in poor condition, died after treatment. Seven of the treated animals were slaughtered three weeks after treatment; six had no flukes in the livers while the seventh cow had one live immature fluke.

b) *Dosage: One capsule to 70 lb. of body weight, administered in three and four days.*

Twenty-eight young beef animals, weighing from 176 to 770 lb., were treated. Of these, 14 received the drug over three days and 14 over four days; the total amount of drug given to these animals ranged from three to eleven capsules. Following treatment, of those receiving the drug in three days, ten were positive for fluke eggs and four were negative. Of those treated in four days, six were positive and eight were negative.

3) TREATMENT WITH HEXACHLOROETHANE

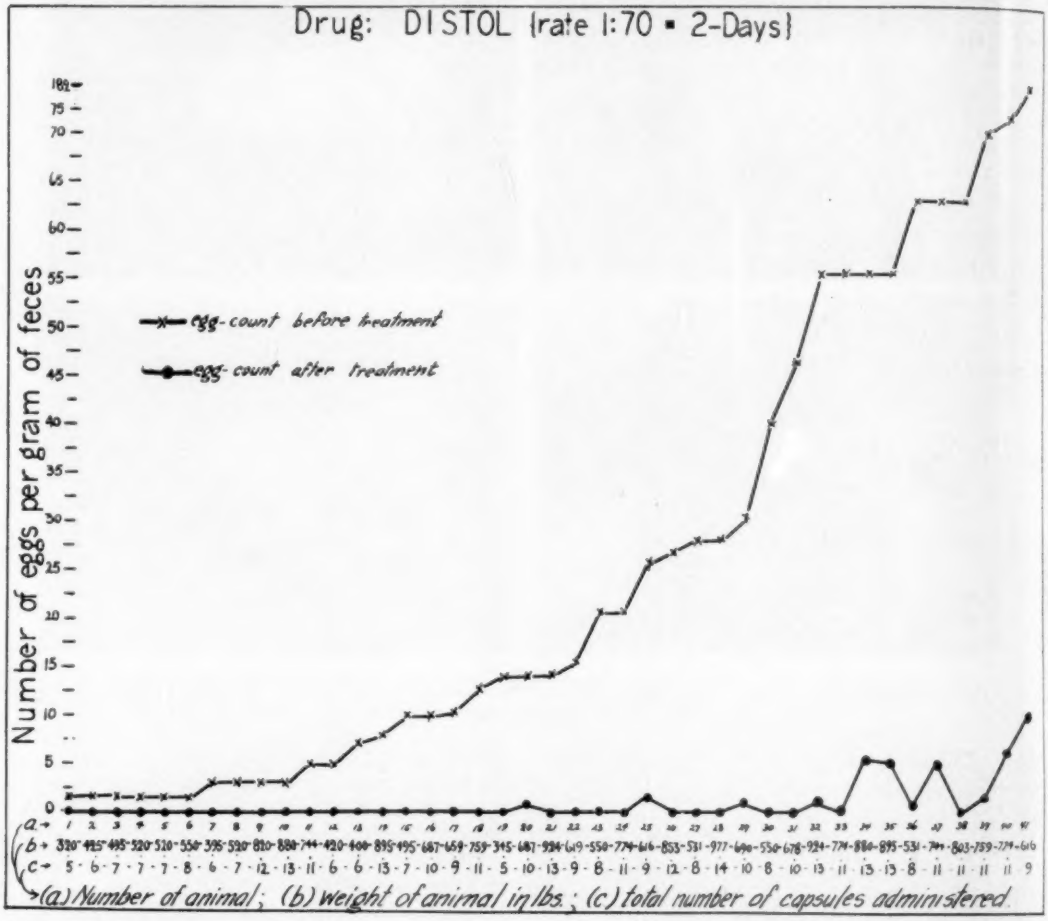
a) *Dosage: One capsule to 60 lb. of body weight, administered in two, three and four days.*

Two cows weighing 1,005 and 1,010 lb., with egg counts of 9.8 and 29.4 per gram of feces, received a total of 17 capsules each (each capsule containing 10 Gm. of the drug), administered over a two-day period. At slaughter two weeks later, one cow was negative for flukes and the other showed one live immature fluke. A third animal, weighing 1,096 lb., developed colic after the administration of half of the dosage (nine capsules).

Another three cows, weighing 1,016, 1,138 and 900 lb., with egg counts of 12.6, 173.6 and 4.2, respectively, received 17, 19 and 15 capsules, respectively, over a three-day period. After treatment two cows were negative; the one which had the highest egg count remained positive, but the egg count had been reduced to 9.8 eggs per gram of feces.

Another three cows, weighing 1,095, 750 and

TABLE I—Effect of Distol on Cattle Showing Various Degrees of Liver-Fluke Infection; Drug Administered at Rate of One Capsule to Each 70 Lb. of Body Weight Over Two Successive Days



820 lb., with egg counts of 8.4, 7 and 3.5, respectively, received 18, 12 and 14 capsules, respectively, over a four-day period. After treatment all the animals were negative for flukes.

A cow weighing 915 lb. was to be given the drug at the rate of one capsule to 70 lb. of body weight over a two-day period. After the first half of the treatment, consisting of six capsules, the cow developed colic, and the second dose was not given.

The above results point out that hexachloroethane was highly effective in most instances in destroying flukes, but in several instances the cows did not tolerate the drug at high concentrations.

4) TREATMENT WITH HEXACHLOROETHANE AND KAMALA EXTRACT COMBINED

a) *Dosage: One capsule to 70 lb. of body weight, administered in two days.*

As indicated above, hexachloroethane, when used alone in heavy concentrations, has a tendency to cause colic. This drug was combined, therefore, with kamala extract; the value of this preparation as a flukecide and its effect on animals were determined experimentally. After a few satisfactory trials, capsules were prepared, each containing a mixture of 10 Gm. of hexachloroethane and 1.75 Gm. of kamala extract. Twenty-six cows were treated at the rate of 1:70, administered over a two-day period. The animals ranged in weight from 684 to 1,090 lb., and the total number of capsules administered to each animal varied from 10 to 16.

The results, which are given in table III, show that in cases where the egg count was below 35 eggs per gram of feces, this dosage completely eliminated all adult flukes, as evidenced by subsequent absence of fluke eggs from the feces. For heavier infections the drug was not as consistent, but the number of fluke eggs was drastically reduced, which indicated the elimination of many adult flukes.

One of the animals, slaughtered two days

after the treatment ended, had several dead and degenerating flukes in the bile ducts.

Most of the 26 cows treated with the drug showed no ill effects; two of the animals, which were in very poor condition when treated, appeared weak and dull shortly after the treatments. It is probably more desirable to treat animals in poor condition over a three-day period.

DRUG TREATMENT WITH REFERENCE TO GESTATION, MILK PRODUCTION AND GENERAL EFFECT ON THE PHYSICAL CONDITION OF THE ANIMAL

Many cows in various degrees of gestation were treated; no undue ill effects were noted even in a few of the cows treated within about two months and one month before calving. Experimental evidence is not sufficient, however, for recommending the treatment of cows during the last two months of lactation.

Most of the cows which have received treatment have been either nonlactating or near the end of their lactation period. In the lactating cows a temporary drop in milk production usually occurred. When treated with distol, the milk acquired a slightly salty bitter taste which lasted a few days. With three cows treated with

TABLE II—Effect of Kamala on Cattle Showing Various Degrees of Liver-Fluke Infection; Drug Administered at Rate of One Capsule (10 Gm.) to Each 60 Lb. of Body Weight Over Four Successive Days

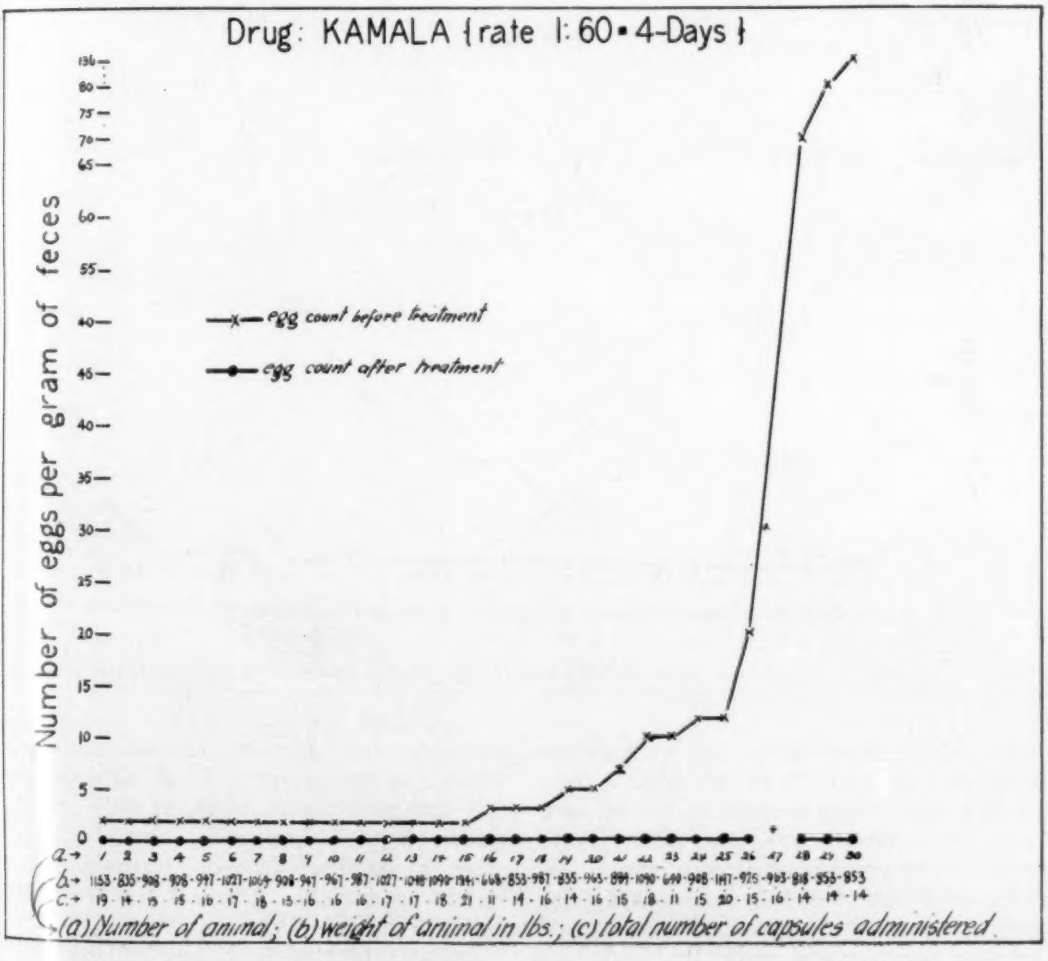
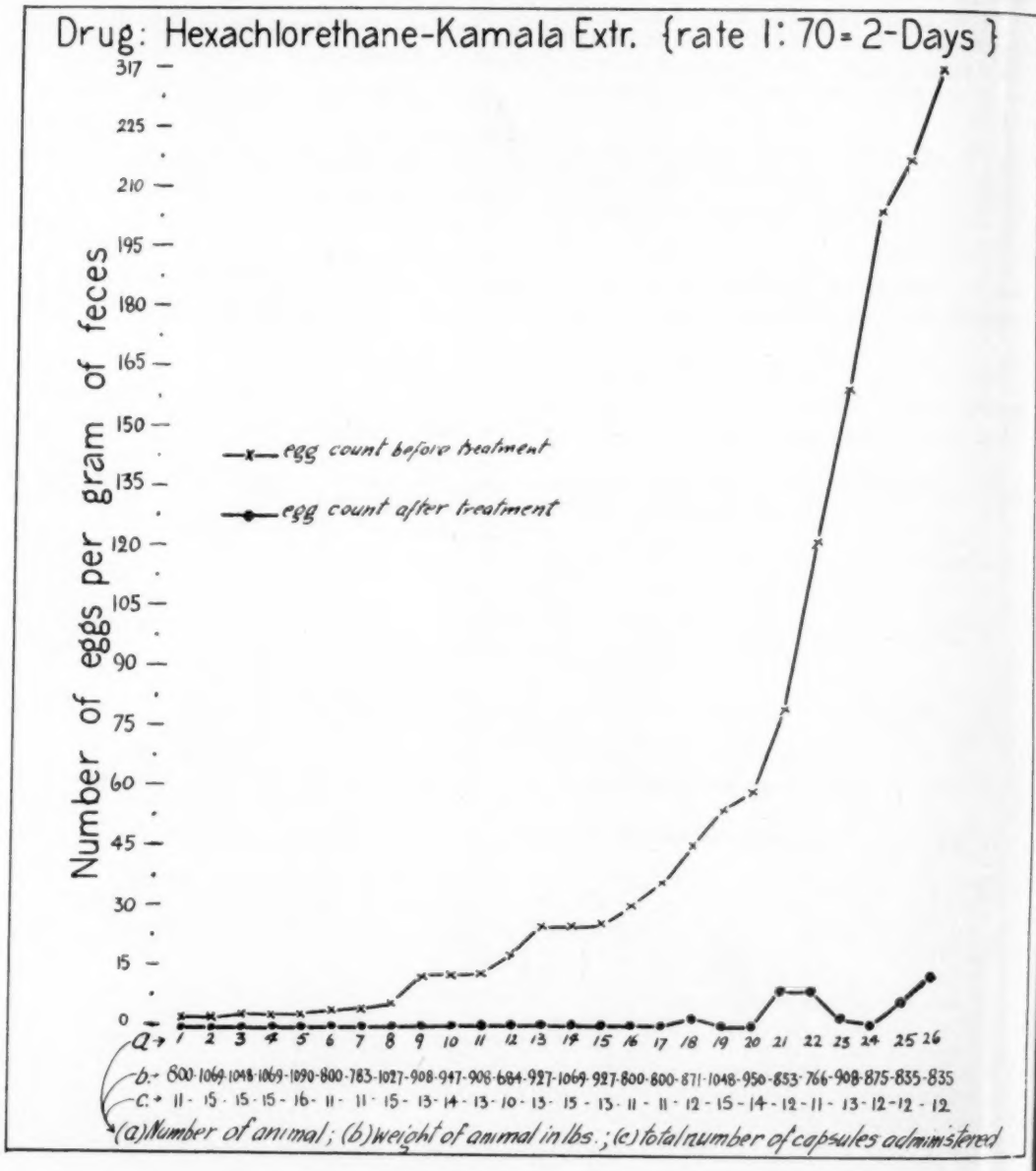

Drug: KAMALA {rate 1:60 • 4-Days}
Number of eggs per gram of feces
egg count before treatment
egg count after treatment
(a)Number of animal; (b)weight of animal in lbs.; (c)total number of capsules administered.

TABLE III—Effect of Hexachloroethane Combined with Kamala Extract on Cattle Showing Various Degrees of Liver-Fluke Infection; Drug Administered at Rate of One Capsule (11.75 Gm.) to Each 70 Lb. of Body Weight Over Two Successive Days



distol, which were giving an average of about 42, 30, and 12 lb. of milk a day, the milk production dropped to 31, 16, and 7 lb., respectively, one day after treatment; on the succeeding days, however, the milk production gradually increased and in about 2 weeks, the production was normal. Another cow, treated with the hexachloroethane and kamala extract mixture, which was giving about 15 lb. of milk before treatment, gave 10 lb. of milk on the second day of treatment; about a week after treatment, the milk production returned to normal. The results are summarized in table IV.

Animals treated with any of the four

drugs showed considerable general physical improvement, as is evidenced by increase in weight and a smoothness and shininess of the hair (see figs. 1, 2, and 3).

ESTIMATING THE DEGREE OF LIVER-FLUKE INFECTION BY THE EGG-COUNT METHOD

Egg counts in helminthiasis, made from definite weighed samples of feces, have been found useful in some cases in determining the number of worms present in the host, and have also aided in determining the efficacy of anthelmintics when made before and after treatment. In connection with studies on the effect of various drugs on the liver fluke of cattle, it was believed desirable to determine whether or not such counts were reliable in liver-fluke infections. After several trials of various known methods, a sedimentation method was devised which has been adopted throughout the experiments on drug treatment.

The method, aimed to determine the number of fluke eggs per gram of feces, was as follows: A sample of about 300 Gm. of fresh feces was obtained from each cow. The feces were then mixed with a glass rod, and a 15-Gm. sample was weighed out and emulsified with about 200 cc. of 4 per cent solution of sodium hydroxide. This mixture was stored overnight in a refrigerator in an Erlenmeyer flask. The following day the feces were shaken thoroughly with the aid of glass beads, tap water was added, and the mixture was poured through three sieves of 20-, 40- and 60-to-the-inch mesh in turn. The feces were thoroughly washed while passing from one sieve to another; the washing, which usually amounted to about 3,000 cc., was retained in a large beaker, and the fluke eggs were allowed to settle overnight.

TABLE IV—Effect of Distol and of Hexachloroethane Combined with Kamala Extract on Milk Production of Dairy Cows

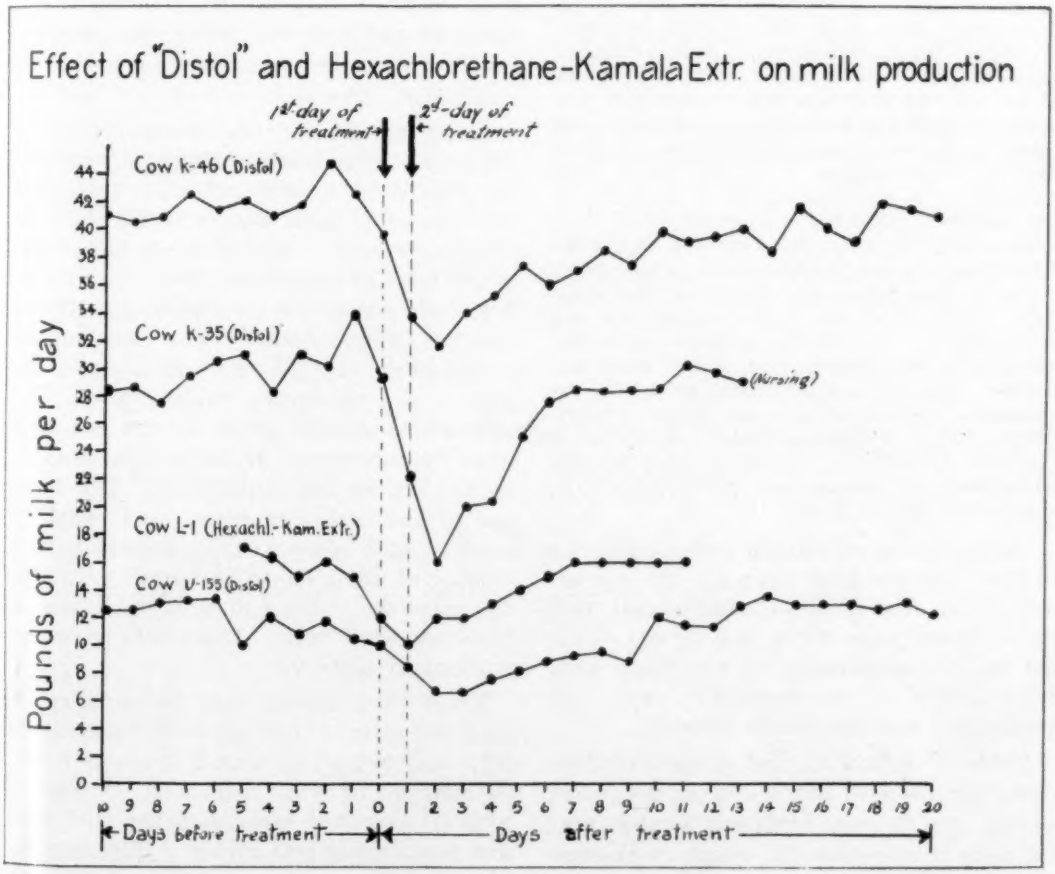


TABLE V—Fluke-Egg Counts per Gram of Feces of Ten Cows for Ten Consecutive Days

Day of Examination	Cow Number									
	1	2	3	4	5	6	7	8	9	10
1st	4.6	5.6	7.0	5.6	9.3	10.5	18.6	37.8	67.2	57 4
2nd	3.5	5.6	4.2	8.4	7.0	10.5	17.5	65.8	53.2	65 8
3rd	7.0	5.6	8.4	5.6	5.8	7.0	14.0	43.4	60.2	29.4
4th	7.0	4.2	5.6	7.0	5.8	7.0	15.1	68.6	47.6	44.8
5th	7.0	4.2	4.2	7.0	9.3	8.1	14.0	40.6	53.2	33.6
6th	5.8	5.6	4.2	8.4	5.8	7.0	14.0	43.4	29.4	60.2
7th	3.5	7.0	7.0	5.6	7.0	10.5	14.0	33.6	29.4	70.0
8th	4.6	4.2	4.2	9.8	9.3	9.3	16.3	33.1	42.0	67.8
9th	7.0	8.4	8.4	7.0	7.0	7.0	15.1	43.4	58.8	39.2
10th	3.5	4.2	5.6	5.6	5.8	5.8	17.5	61.2	None taken	57.4
Mean	5.35	5.46	5.8	7.0	7.21	8.27	15.6	47.13	49.0	52.56
Standard error—										
If one sample was taken	1.58	1.39	1.79	1.48	1.54	1.78	1.74	13.92	13.36	14.68
If two samples were taken	1.26	0.91	1.27	1.05	1.09	1.26	1.23	9.37	9.4	10.41

On the following day, the supernatant fluid was siphoned off to within 1 in. of the bottom of the container, and additional tap water was added to wash the residue; the eggs were allowed to resettle overnight. The supernatant fluid was again siphoned off, the sediment placed in a graduated cylinder, and enough tap water added, if necessary, to reach the volume of 105 cc. The fluid was than transferred to a 300-cc. Erlenmeyer flask, and an egg count was made as follows: Immediately after the fluid had been well shaken, 1 cc. was drawn with a calibrated pipette and spread in long narrow streaks on a glass slide approximately 7 in. long and 3.5 in. wide. With the aid of a wide-field Spencer binocular microscope, a count was made of the fluke eggs found on the slide. After the eggs of five such preparations had been counted, the average number was taken to represent the number of eggs per cubic centimeter. This average multiplied by 7 gave the estimated number of eggs per gram of feces. (The number of eggs per cubic centimeter of the 105-cc. mixture represents theoretically one-seventh of the number per gram of the original 15 Gm. of feces.)

Before the egg-counting method could be utilized for practical purposes, it was essential to determine to what extent this count would vary from day to day. This led to the examination of ten fluky cows for a period of ten successive days; the results are summarized in table V.

Table V indicates that some variation does take place in the egg count from day to day, and in some instances a count may be more than double the count on another day. From the standpoint of practical application of this method in drug treatment, if only one count is made before treatment, it is essential that the count after treatment be reduced well below the possible range of error before the drug can be considered effective.

Additional studies were also conducted to determine a possible correlation between the number of eggs found in the feces and the number of adult worms in the livers of infected animals. Two egg counts were made of the feces of each of ten fluky cows a few days before they were slaughtered. At the time of slaughter the liver of each animal was removed, and a count was made of the mature and immature flukes present. In addition, a sample of feces was collected from the lower part of the rectum of each animal and an egg count made. The average of the three-egg counts per gram of feces of each cow was then divided by the number of adult flukes harbored by it, and this gave the number of eggs per gram of feces per adult fluke. These data are summarized in table VI.

Table VI indicates that the number of eggs per gram of feces per adult fluke varies from 0.47 to 1.40, with an average of 1.013. Calculation of the regression coefficient with its standard error demonstrated that this relationship was highly significant. On

this basis, by dividing the egg count of animal by the above average, it appears possible to estimate the general index of adult flukes found in infected cattle as well as the efficacy of the drug when counts are made before and after treatment.

It is also of interest to point out that 36 additional cows were examined at slaughter and the number of adult flukes present and eggs per gram of feces, based on a single specimen, determined. The average of these egg counts was found to be 1.028 (also statistically significant), which, in spite of the fact that a single count was made, is very similar to the average obtained from three counts made on each of the ten cows listed in table VI.

SUMMARY AND CONCLUSIONS

The problem of liver flukes in cattle in the Hawaiian Islands is being attacked from the standpoint of (a) controlling the fresh-water snails *(Fossaria ollula)*, which serve as intermediate hosts; (b) protecting the animals from infection; and (c) anthelmintic treatment of infected animals.

Fresh-water snails may be controlled by the established methods of applying copper sulfate in infested areas and drainage. In addition, fireflies have been advocated by the writer as a possible biological means of controlling the snails; the larvae of these insects are carnivorous and utilize fresh-water snails and other soft-bodied invertebrates as food. The Territorial Board of Commissioners of Agriculture and Forestry has introduced fireflies (Luciola spp.) into the Islands from Japan and the Philippines. These insects are now being reared and are being liberated in suitable localities; whether or not they will become established remains to be determined.

For the protection of animals from infection, it is recommended that infected areas be fenced off and that animals should not be fed vegetation cut from these regions. The writer has also suggested ensilage as a possible means of destroying the encysted flukes (metacercariae) on vegetation. This suggestion is based on preliminary experiments; additional experiments along these lines will be necessary, however, before definite conclusions can be drawn.

Several preparations seem to be suitable for the destruction of flukes in cattle. Distol, a proprietary drug, has been found highly effective in destroying flukes and is well tolerated by the animals. Satisfactory results have been obtained with distol at the rate of one capsule to each 70 lb. of body weight, the total amount divided into equal or approximately equal dosages and administered over a period of two successive days. Kamala has also been found effective in destroying liver flukes when administered at the rate of one capsule (10 Gm.) to each 60 lb. of body weight, over a four-day period. The latter drug, however, has been found to cause extensive diarrhea

TABLE VI—Fluke and Fluke-Egg Count Made of Ten Adult Cows

Number of Animal	Number of Flukes in Liver		Fluke-Egg Count per Gram of Feces				Estimated Number of Eggs per Gram of Feces per Mature Fluke
	Mature	Immature	Sample 1	Sample 2	Sample 3	Average	
1	4	0	2.8	1.4	2.8	2.33	0.58
2	5	0	1.4	2.8	2.8	2.33	0.47
3	76	5	50.4	128.8	131.6	103.60	1.36
4	6	6	8.4	5.6	8.4	7.46	1.24
5	19	3	21.0	16.8	14.0	17.26	0.91
6	52	4	43.4	61.6	33.6	46.20	0.88
7	20	0	18.2	28.0	21.0	22.40	1.12
8	11	0	12.6	12.6	18.2	14.47	1.31
9	2	0	2.8	1.4	4.2	2.80	1.40
10	21	0	17.5	14.0	22.4	17.97	.86

Mean 1.013

and loss of appetite, lasting for a few days to about two weeks.

Hexachloroethane also appears to be effective in destroying flukes, but it has been found to cause colic when given at high concentrations. The writer has found this drug to be well tolerated and highly effective when mixed with kamala extract and given at the rate of one capsule (10 Gm. hexachloroethane and 1.75 Gm. kamala extract) to each 70 lb. of body weight over two successive days. Twenty-six cows have been treated successfully with this new preparation.

Most of the animals which have been treated with distol, kamala, or hexachloroethane and kamala extract combined, have improved in general physical condition. A few of the lactating cows treated have shown a slight decrease in milk production following treatment, lasting from one to two weeks.

An egg-count method has been described for determining the fluke-egg output per gram of feces of fluky cattle. The efficacy of the various drugs has been established by making counts before and after anthelmintic treatment. In addition, a study has been made of the relationship between the number of eggs per gram of feces in ten fluky animals and the number of flukes harbored by them when slaughtered a few days later. The average number of eggs per gram of feces was found to be 1.013 per adult fluke. On this basis it is possible to estimate the approximate number of adult flukes harbored by an infected animal.

Corrections

In the article by Grey, Osteen and Schoening, entitled "Swine Erysipelas, The Agglutination Test for Its Diagnosis, and a Report on a Study of Arthritis in Swine," which appeared in the January 1941 issue, the date "1932" on page 76, column 2, paragraph 1, line 18, should read "1939."

In the article by Nolan and Hull, entitled "Grass Tetany in Cattle," which appeared in the January 1941 issue, the sentence "Of the twelve cases we have studied eleven occurred during the winter when the animals were almost entirely on poor quality hay" on page 41, column 2, paragraph 1, line 25, should read "Eleven of the twelve cases we have studied were fed almost entirely on poor quality hay during the winter months."

The Hereditary Transmission of Anaplasmosis by Dermacentor Andersoni Stiles*

D. E. HOWELL, B.S., M.S., Ph.D., GEO. W. STILES, B.S., M.D., Ph.D., and LEWIS H. MOE, D.V.M., M.Sc.

DIKMANS[1] suggests that the hereditary transmission of Anaplasma bodies by ticks was first experimentally demonstrated by Smith and Kilborne[2] during the course of their transmission experiments with tick fever of cattle. *Boophilus annulatus* (Say) was the tick vector.

Theiler[3, 4] found that *B. decoloratus* (Koch) and *Rhipicephalus simus* Koch larvae carried Anaplasma bodies when they hatched from the egg if their parent had fed on an animal suffering from anaplasmosis. *B. microplus* Neum.,[5, 6] *B. australis* Fuller,[7] *Ixodes ricinus* Linn.,[8] *Hyalomma lusitanicum* Koch,[9] *B. annulatus* (Say)[10] and *Dermacentor occidentalis* Neum.[11] have been subsequently incriminated as hereditary vectors. Rees,[11] Boynton *et al*[10] and Rozeboom *et al*[12] reported successful stage-to-stage transmissions with *D. andersoni*, but failed to obtain any evidence of hereditary transmission. Sanborn *et al*[13] reported a probable successful hereditary transmission. Ticks collected in Wyoming from a clinical case of anaplasmosis were shipped to the Oklahoma station and allowed to reattach and feed to repletion on an animal with anaplasmosis. The offspring of these ticks were fed on a normal animal which developed "forms in the erythrocytes suggestive of anaplasmosis. However, the animal died of accidental strangulation before the opportunity was afforded to test her as a carrier."

PRESENT WRITERS' EXPERIMENTS

On May 2, 1940, one of the authors (G.W.S.) collected several adult *D. andersoni* ticks from a naturally infested cow in Wyoming during the initial stages of anaplasmosis. Her temperature was 102.6° F. and her hemoglobin, 65. Blood smears showed marginal bodies in the erythrocytes. One of the ticks was replete and apparently just ready to drop from the host; the others were between one-fourth and one-half replete. These were sent to the Oklahoma station and used as described below.

Case 255.—Eggs were first deposited by the replete tick June 2, and started hatching July 1. July 12, the resulting larvae were placed in a small sack that was securely tied and taped to the ear of cow 255, an animal purchased several weeks previously at a commu-

*This paper reports a coöperative project conducted by the Oklahoma Agricultural Experiment Station and the Bureau of Animal Industry, U. S. Department of Agriculture. Representing the former are D. E. Howell, assistant professor of medical entomology, and Lewis H. Moe, associate professor of veterinary science. The Bureau of Animal Industry is represented by Geo. W. Stiles, bacteriologist in charge of the Branch Pathological Laboratory, Denver, Colo.

[1]Dikmans, G.: Anaplasmosis. IV. The carrier problem. J.A.V.M.A., lxxxii (1933), pp. 862-870.

[2]Smith, T., and Kilborne, F. E.: Investigations into the nature, causation and prevention of Texas or southern cattle fever. U. S. Dept. Agr., Bur. Ani. Ind. Bul. 1 (1893), 301 pp.

[3]Theiler, A.: *Anaplasma marginale* (gen. and spec. nov.). The marginal points in the blood of cattle suffering from a specific disease. Transvaal Dept. Agr., Rpt. Gov. Vet. Bact., 1908-09 (1910), pp. 1-73.

[4]*Ibid.*: Übertragung der Anaplasmosis mittels Zecken. Zeit. Infectionsk. u. Hyg. Haust., xii (1912), pp. 105-116.

[5]Rosenbusch, F., and Gonzales, R.: Garrapatización y tristeza. Investigaciones experimentales. (11A communicación.) Ann. Soc. Rural Argentina, lvii (1923), pp. 789-799.

[6]*Ibid.*: Die Tristeza-Übertragung durch Zecken und dessen Immunitätsprobleme. Arch. Protistenk., lviii (1927), pp. 300-320.

[7]Brumpt, E.: Transmission d'*Anaplasma marginale* par *Rhipicephalus bursa* et par Margaropus. Ann. Parasitol. Humaine et Comp., ix (1931), pp. 4-9.

[8]Helm, R.: Beitrag zum Anaplasmen-problem. Zeit. Infektionsk. u. Hyg. Haust., xxv (1924), pp. 199-226.

[9]Sergent, E., Donatien, A., Parrot, L., and Lestoquard, F.: Tiques et piroplasmoses bovines d'Algérie. Première note. Bul. Soc. Path. Exot., xxi (1928), pp. 846-849.

[10]Boynton, W. H., Herms, W. B., Howell, D. E., and Woods, G. M.: Anaplasmosis transmission by three species of ticks in California. J. A. V. M. A., lxxxviii (1936), pp. 500-502.

[11]Rees, C. W.: Transmission of anaplasmosis by various species of ticks. U. S. Dept. Agr. Tech. Bul. 418 (1934).

[12]Rozeboom, L. E., Stiles, G. W., and Moe, L. H.: Anaplasmosis transmission by *Dermacentor andersoni*. J. Parasitol., xxvi (1940), pp. 95-100.

[13]Sanborn, C. E., Stiles, G. W., and Moe, L. H.: Anaplasmosis transmission by naturally infected *Dermacentor andersoni* male and female ticks. No. Amer. Vet., xix (1938), pp. 31-33.

nity sale and subsequently kept in the isolation barn. Twelve days later many replete larvae were removed and kept for further work. The ear was carefully dusted with concentrated derris powder to kill any larvae that might remain unseen.

Blood smears taken from this animal August 20, 39 days after the ticks were applied, showed numerous Anaplasma bodies. The animal refused feed, showed a temperature of 104.8° F., accelerated respiration and pulse, and walked with difficulty. She made a slow, uneventful recovery and has been used as a carrier in subsequent transmission work.

Case 254.—The remaining ticks of the Wyoming lot were placed in the ear of calf 245 and allowed to remain until June 10. This animal showed numerous marginal bodies, but no clinical symptoms of anaplasmosis.

Eggs were first deposited June 22 and hatching began July 19. The resulting larvae were placed in the ear of cow 254 a week later and allowed to remain until August 21. At this time many replete larvae were removed, though a few still remained unfed. The ear was treated to prevent the escape of any ticks that were not collected.

Despite the large number of larvae used and the apparently favorable conditions for transmission, blood smears have remained consistently negative.

DISCUSSION

It is interesting to note that a single case of hereditary transmission of anaplasmosis by *D. andersoni* has been accomplished despite repeated failures by competent investigators (Rees,[11] Rees and Avery[14] and Rozeboom *et al*[12]). This case takes on added interest when it is noted that the parent of the larvae was infected by feeding on an animal which was just beginning to show clinical symptoms of anaplasmosis. Is it possible that *D. andersoni* can be infected most readily by feeding on animals in the initial stage of the disease? Further work is being carried on to test this hypothesis.

Rees and Avery have pointed out that *D. andersoni* can have little importance in spreading anaplasmosis, as the immature stages seldom feed on cattle. It is well known, however, that *D. andersoni* is the only proved vector found in large numbers in much of the area occupied by anaplasmosis; hence, one is forced to conclude that hereditary transmission may be of some importance or that some unknown factor is responsible for the spread of the disease in large areas of the Northwest.

SUMMARY

A successful transmission of anaplasmosis by the progeny of a naturally infected *Dermacentor andersoni* female tick is recorded.

A second animal on which *D. andersoni* larvae from a similar source engorged failed to develop the disease.

[11]Rees, C. W., and Avery, J. L.: Experiments on the hereditary transmission of anaplasmosis by ticks. No. Amer. Vet., xx (1939), pp. 35-36.

The pH of Vaginal Mucus of Normal and Sterile Dairy Cows*

SEDGWICK E. SMITH, Ph.D., and S. A. ASDELL, Ph.D.
Ithaca, N. Y.

ACIDITY of the vaginal tract is not infrequently cited as a cause of sterility. A douche of sodium bicarbonate is often recommended just prior to service to neutralize the tract, thus creating a more favorable environment for the deposited spermatozoa. In a study of the etiology and therapy of functional sterility in dairy cows it was of interest to determine the vaginal pH of such cows. As this work progressed, knowledge of the pH of normal vaginal tracts was required in order to evaluate our findings in the sterile cows. The results of these and allied investigations are reported here.

REVIEW OF LITERATURE

The association of vaginal acidity and sterility probably arose in the field of gynecology. Meaker and Glasser[1] cited Jackson, who in 1877 wrote, "The most frequent cause of untimely death of spermatozoa is the acid mucus of the vagina." That the human female vaginal tract is acid under average conditions has been shown by many (Demme,[2] Kessler and Rohrs,[3] Behrens and Naujak[4]). Since a pH below 6.0 causes a cessation of sperm motility in man (Muschat[5]), it can be seen that vaginal pH may be an important factor in fertility in women. However, the work of Meaker and Glasser shows that vaginal acidity does not play a large rôle in human sterility.

While many looked upon vaginal acidity as playing a major rôle in bovine sterility, some did not, as is shown by the statement of Reinhardt,[6] "The frequently held view that in disease the vaginal secretion is acid and that this condition has an especially deleterious effect on sperm, is not true, for in normal as well as in diseased vaginas the reaction for the most part is neutral or alkaline." Wester[7] attributed favorable results to sodium bicarbonate douches in sterile animals, but unlike others who thought in terms of a neutralization of harmful acids, he thought it was due to the mechanical removal of "spermatoxins" arising from the existent infections. Webster had found that normal vaginal contents were alkaline to litmus paper. Kaden[8] made an extensive survey of the vaginal reactions in 84 live and 39 slaughtered animals, using litmus paper or phenolphthalein. Of 190 vaginal samples studied, 189 were alkaline and one acidic. Of 23 diseased animals 19 had alkaline reactions. He cited Renkert and Find, who found nonacidic reactions in cows that had (1) aborted, (2) secondary placental retention or (3) endometritis. Kaden came to the conclusion that an acid reaction of the vagina is not an important factor in sterility of cattle.

Woodman and Hammond[9] studied vaginal and cervical mucus in 30 slaughtered cows and in no case was an acid reaction obtained with litmus paper. Whereas the cervical mucus showed a feeble alkaline reaction, the vaginal mucus showed a stronger alkaline reaction. Since the vagina is alkaline and dilute alkaline solutions dissolve mucus, they suggest that the thin, streaming mucus of estrus is due to the contact of the cervical mucus with the more alkaline vagina. Thus the cervical canal is opened for the passage of spermatozoa.

Since this work was undertaken, McNutt, Schwarte and Eveleth[10] published work on the pH of vaginal secretions of normal heifers and cows. Their results, obtained on a vaginal saline wash with a glass electrode, showed the vaginal tract to fluctuate around neutrality and to be on the alkaline side at estrus. They

*From the Laboratory of Animal Nutrition, Cornell University.

[1]Meaker, S. R., and Glasser, W.: The hydrogen ion concentration of the endocervical secretions. Surg., Gynec. & Obst., xlviii (1929), p. 73.

[2]Demme, R.: Bakteriologische-biologische Studien der in der Vagina vorkommenden Mikroorganismen und ihrer Beziehungen zum Selbetreinigungvermogen der Scheide. Arch. Gynak., cxxix (1927), p. 900.

[3]Kessler, R., and Rohrs, H. D.: Scheidenbiologische Studien an Neugeborenen, Sauglingen und Kleinmadchen. Arch. Gynak., cxxix (1927), p. 856.

[4]Behrens and Naujak: The degree of acidity of the vaginal secretion. (title trans.) Zeit. ges. exp. Med., xlvii (1925), p. 178.

[5]Muschat, M.: The effect of variations of hydrogen ion concentration on the motility of human spermatozoa. Surg., Gynec. & Obst., xlii (1926), p. 778.

[6]Reinhardt, R. H.: Lehrbuch der tierärztlichen Geburtschilfe (Berlin, 1920).

[7]Wester, J.: Eierstock und Ei (Berlin, 1921).

[8]Kaden, H. W.: Untersuchungen über die chemische Reaktion des Vaginalsekretes beim Rind. Inaug. Diss. (Leipzig, 1921).

[9]Woodman, H. E., and Hammond, J.: The mucous secretion of the cervix of the cow. J. Agr. Sci., xv (1925), p. 107.

[10]McNutt, S. H., Schwarte, L. H., and Eveleth, D. F.: The hydrogen ion concentration of vaginal secretions of cows. Cornell Vet., xxix (1939), p. 415.

also stated that "secretions of the cervical canal appear more alkaline than the vagina." Our pH values range higher than those reported by the aforementioned workers and the probable reason for the discrepancy is pointed out later. Our results contradict the idea that cervical mucus is more alkaline than vaginal mucus.

The only other farm animal whose vaginal pH has been carefully studied is the mare. Dybing,[11] using a colorimetric method, found the vaginal mucus of mares slaughtered at a packing plant to average about pH 7.6. The cervical mucus averaged about pH 6.9 and was thus more acid than vaginal mucus. Andrews[12] is essentially in agreement with Dybing. Using the more accurate glass electrode, he found the vaginal mucus in over 50 normal mares to range about pH 8.1 and the cervical mucus about pH 7.8. Andrews also found that at five to ten days following estrus, the vaginal pH was at its lowest.

Kardymovic[13] found that the pH of the vaginas of ewes varied with the estrous cycle, being more alkaline at estrus and becoming more acid as the cycle progresses.

MATERIALS AND METHODS

The normal animals studied were mature, Bang-, trichomonad- and tuberculosis-free Holstein cows kept in the herd of Cornell University. They had calved and were experiencing regular estrous cycles. Rectal and vaginal examinations showed an absence of infections and abnormalities. The functionally sterile cows were part of a herd collected from New York state breeders that were being treated with various endocrine preparations in an attempt to restore fertility. These cows tested free of Bang's disease, trichomonads and tuberculosis. Vaginal and rectal examinations failed to reveal a cause of sterility, yet the cows failed to conceive when mated to a bull of known fertility. These cows for the most part were running fairly regular estrous cycles. Some nymphomaniac (persistent estrus) and some anaphrodisiac (absence of estrus) cows also were studied. A few miscellaneous cows were studied at the Pennsylvania State College and in a private herd. A total of over 30 cows were studied, some of which were followed through a period of six to eight months.

Except as otherwise noted, mucus was obtained manually from the vagina, care being taken to have clean hands that were well coated with petrolatum. Little trouble was experienced in getting 1- to 2-Gm. samples. Occasionally at the mid-cycle, amounts less than this had to suffice. The mucus was put immediately into a stoppered glass vial and the pH determined within the hour in the majority of cases.

The pH was determined with a glass electrode, a Beckman pH meter being used. The accuracy and adaptability of the glass electrode to biological material make it especially useful. All work on the vaginal secretions of cattle heretofore with the exception of that done by McNutt and coworkers was done with very rough pH indicators, such as litmus paper and phenolphthalein.

The Beckman meter was checked before and after each run of analyses with a standard buffer solution. It was found that the first two or three readings were usually off, but following this the glass electrode became stable and successive readings on a sample of mucus could be checked within 0.2 of a pH unit provided that time was allowed for an equilibrium to be established. This often took 15 to 20 minutes. This drift in potential was not due to the escape of carbon dioxide, for the initial reading was invariably more alkaline than the final reading. It is felt that the pH values reported are correct within 0.2 pH units. In all cases average pH values are arithmetic averages.

The estrogenic hormone used on some of the cows was progynon-B.* Various amounts of the hormone were injected subcutaneously in the neck.

RESULTS

Since the results of McNutt *et al* showed the pH of the vagina of normal cows and heifers to range approximately a pH unit less than ours, it became important to determine, if possible, the cause of this discrepancy. Our technics differed and it appeared that this might be the cause of the difference. In our series the pH of vaginal mucus was determined, whereas McNutt *et al* determined the pH of a 50-cc. physiologic saline vaginal wash. Five cows were chosen for a comparison of these two methods. In each case a mucus sample was secured manually. Immediately following, 50 cc. of physiologic saline was injected deep into the vagina. At the end of two minutes a sample was withdrawn by means of a glass syringe and put into a glass vial

[11]Dybing, O.: Preliminary observations on the acidity of the genital tract of the mare. (title trans.) Ann. Breed. Abst., v (1937), p. 260.

[12]Andrews, F. N.: Estrus and related phenomena in the mare (Thesis, Univ. of Missouri, 1939.)

[13]Kardymovic, M. K. Vaginal secretion and the sex of progeny in sheep. (title trans.) Ann. Breed. Abst., v (1937), p. 153.

*The authors are grateful to the Schering Corp. for generous amounts of progynon-B.

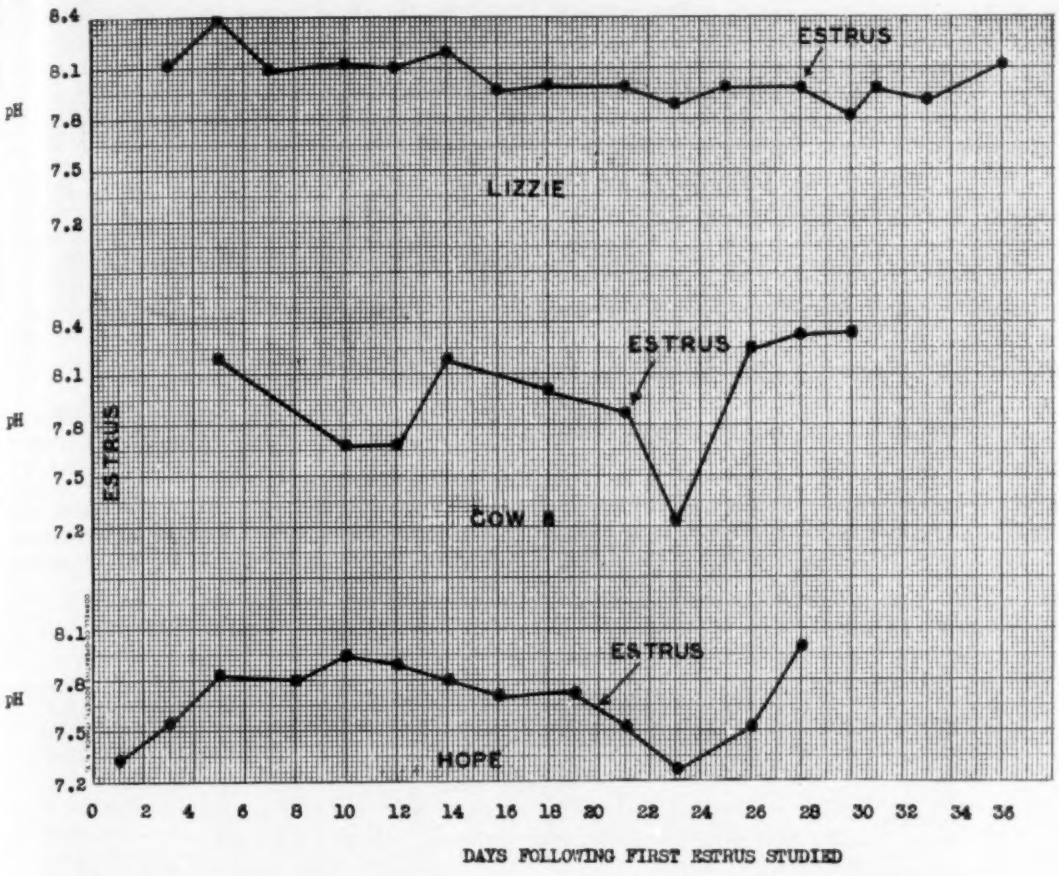


Fig. 1. Changes in the pH of vaginal secretions in normal dairy cows during the estrous cycle.

and stoppered. pH determinations were made of mucus, a saline wash and a 10 per cent solution, with saline as the diluent. The results are summarized in table I.

TABLE I—A Comparison of the pH of Vaginal Mucus and a Vaginal Saline Wash

Cow No.	Vaginal Mucus pH	Vaginal Wash pH	10% Saline Sol. of Mucus pH
29	7.7	7.0	7.2
36	8.1	6.8	7.3
Hope	7.6	7.0	7.0
52	6.6	6.8	6.7
27	7.9	6.8	

From these results it appears that a vaginal saline wash has a pH value 0.5 to 1.0 pH unit less than vaginal mucus when the mucus ranges above pH 7.5. It is likely that this explains the difference in the data obtained by McNutt *et al* and by us. In the rest of our work we report only vaginal mucus pH determinations, as we think that they are the more reliable index of the vaginal reaction.

Figure 1 shows the variation of the pH of the vaginal mucus in three normal cows when determined at two- or three-day intervals throughout the estrous cycle. It will be noted that the pH in all cases was above neutrality; in fact, the average was near pH 8.0. In the case of Hope and No. 8, there was a distinct drop in pH at the second day following estrus. In the case of Lizzie the lowest pH was found at the second day following estrus, but the drop was not marked. The vaginal contents of Lizzie at this time included an abundance of partly decomposed blood absent in the other two cows. Following the postestrous

drop the pH rose to a fairly uniform level until after the next estrus. It is noteworthy that the pH at estrus was still at the high diestrous level.

Figure 2 is a composite graph showing the pH fluctuations of the vaginal mucus during the estrous cycle in five functionally sterile cows. This graph does not differ significantly from the graph of the three normal cows and, therefore, the pH values shown may be considered normal. The pH at estrus averaged 8.0. The day following estrus the pH dropped to a low of 7.2, after which it rose to the diestrous level, ranging from 7.8 to 8.3.

The data in table II are pH values of mucus taken at various intervals from five functionally sterile cows, three of which had vaginal infections and three of which had normal vaginas.

TABLE II—The pH of Vaginal Mucus of Functionally Sterile Cows

Cow No.	pH of Vaginal Mucus	Duration of Study
29*	8.4, 8.0, 7.9, 8.2, 8.4	1 month
27*	8.6, 8.4, 7.3, 7.4	1 month
31*	8.0, 8.3, 8.1, 8.0	1 month
39	8.6, 8.3, 8.2, 8.2	1 month
36	8.0, 7.1†, 7.6, 7.7, 8.1	2 weeks
18	8.4, 8.4, 7.9†, 8.5	3 weeks

*Vaginal infection.
†Third day following estrus.

In none of these animals was a pH below neutrality found. In most cases the pH fluctuated around 8.0. The range was 7.1 to 8.6. There was no significant difference in the vaginal pH of the three cows with infected vaginas and the three cows with normal vaginas.

In table III are given single pH deter-

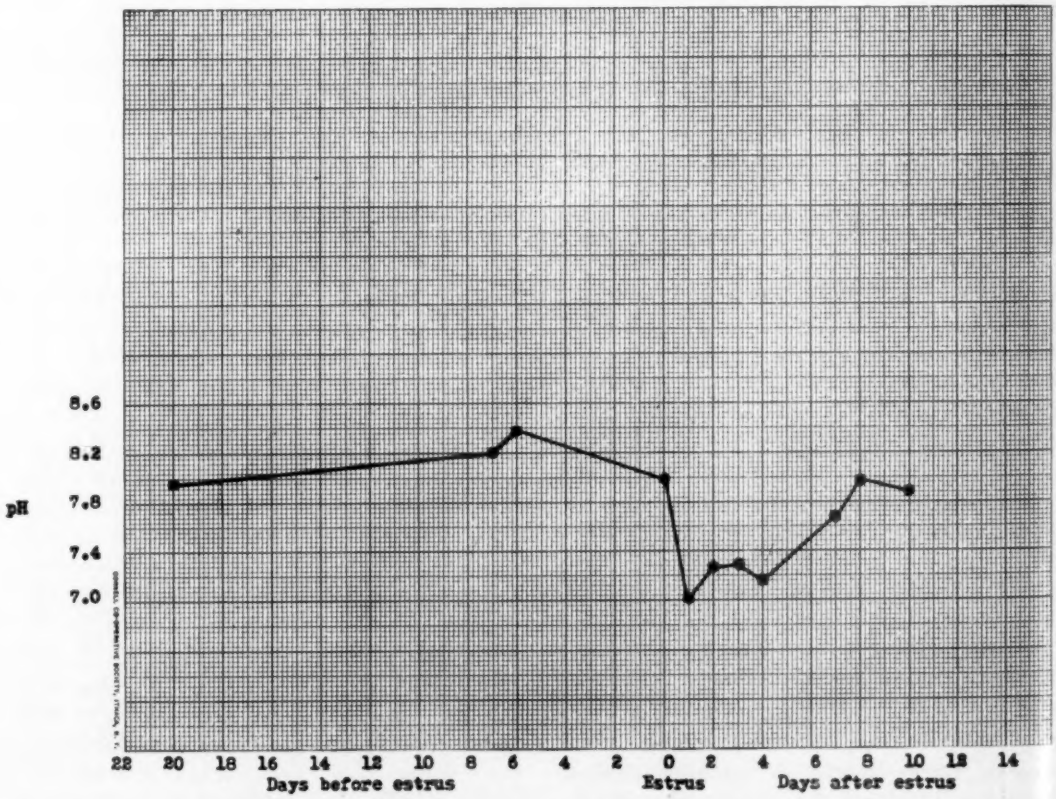


Fig. 2. Changes in the pH of vaginal secretions during the estrous cycle of five functionally sterile dairy cows.

TABLE III—The pH of the Vaginal Mucus of Dairy Cows of Low Fertility

Cow No.	Breed	pH	Remarks
Bella	H	8.1	Absence of estrus for 90 days
Patsy	H	8.4	Will not conceive; regular cycles
May	H	7.9	Will not conceive; regular cycles
Fenco	H	8.6	Long history of sterility, periodic pyometra
Kathleen	H	8.4	Aborted last calf; Bang free
Rose	G	7.0	Nymphomania for a year
155	J	7.1	Difficult breeder
A397	G	7.9	Nymphomaniac
1885	H	7.5	Will not conceive; regular cycles
1390	H	7.7	Will not conceive; regular cycles
43	H	7.1	Absence of estrus for a year
42	H	7.9	Functional sterility; irregular cycles
49	H	6.80 6.1	Pyometra discharging through vagina
51	H	8.0	Vaginal infection; irregular cycles; will not breed
41	H	7.4	Difficult breeder
46	H	7.9	Will not breed

minations made on the vaginal mucus of a miscellaneous group of cows and heifers of low fertility. These samples of mucus were taken without regard to the time of the estrous cycle.

In only one case among this heterogeneous group of cows was the vaginal pH below 7.0. Cow 49 had a pH of 6.8 and 6.1 on different occasions which is probably accounted for by the muco-pus which was discharging through the vagina from an infected uterus.

As a result of this survey we believe that vaginal acidity can not be considered an important cause of sterility in dairy cows.

The pH of the vaginal mucus and cervical mucus was compared in four cows which were functionally sterile and had normal appearing vaginas and cervices. The genital tracts were secured from the animals at slaughter. The vaginas and cervices were cut open and a small amount of mucus was taken from each. The pH was determined on these samples with a microglass electrode. The results of these determinations are summarized in table IV.

In all the animals of this small group the cervical mucus was more acid than the vaginal mucus. This agrees with the observations of Woodman and Hammond which were made with litmus paper.

It has been shown that the injection of estrogenic hormone is of great value in the treatment of children suffering from gonococcus infection (Hall and Lewis,[14] Te Linde,[15] and Lewis[16]). The favorable effect of such treatment has been attributed to a lowering of the vaginal pH. The gonococcus organisms rarely survive below pH 6.0. That estrogenic treatment lowers the vaginal pH in the human female has been shown by a number of workers (Weinstein and Howard,[17] Te Linde[15]).

It was decided to investigate the effects of estrogenic therapy in cattle. Figure 3 shows the pH variations of the vaginal mucus in a cow that was treated with

TABLE IV—The pH of Vaginal and Cervical Mucus from the Same Cow

Cow No.	Vaginal Mucus	Cervical Mucus	Difference
26	8.9	7.0	1.9
23	7.8	6.7	1.1
18	8.8	6.8	2.0
19	8.5	6.6	1.9
Average	8.5	6.8	1.7

[14]Hall, B. V., and Lewis, R. M.: Induction of an acid vaginal secretion in the immature macaque by injection of oestrin. Endocrinol., xx (1936), p. 112.

[15]TeLinde, R. W. The treatment of gonococcic vaginitis with estrogenic hormone. J.A.M.A., cx (1938), p. 1633.

[16]Lewis, R. M.: A study of the effects of theelin on gonorrheal vaginitis in children. Amer. J. Obst. & Gynec., xxvi (1933), p. 593.

[17]Weinstein, L., and Howard, J. H.: The effect of estrogenic hormone on the H-ion concentration and the bacterial content of the human vagina, with special reference to the Doderlein bacillus. Amer. J. Obst. & Gynec., xxxvii (1939), p. 698.

progynon-B at the times marked in figure 3, 10,000 R. U. being given per injection. This cow was selected because she had shown no estrus during the previous 90 days, and if estrus should occur following injections, it would probably be an induced one. Estrus occurred after 50,000 R. U. of progynon-B had been administered. The probability that this estrus was an induced one is additionally supported by the fact that it lasted four days, a time much longer than normal. At the fourth day following the onset of estrus, the vaginal pH dropped to 7.2, a reaction that was typical of a normal cow following estrus. A significant observation, however, is that the pH did not drop below 7.0. Even with the decrease in pH following estrus, the change was not as great as in children treated with estrogens, where values as low as 4.8 have been reported.

Three other cows were injected with a total of 50,000 R. U. each of progynon-B over a period of two weeks. The plotted pH of the vaginal mucus gave a patternless graph so that no significant difference was noted in the pH prior to and following estrus. The estrus which occurred in these three cows may not have been artificially induced. In these three cases a pH below 7.0 was not obtained.

These results with four cows indicate that an acid vaginal reaction can not be obtained by the injection of estrogenic hormone in amounts as high as 50,000 R. U. Cattle thus differ from the human female and the macaque monkey (Hall and Lewis[14]), in which the vagina becomes highly acid after estrogenic treatment.

DISCUSSION

As a result of this study of sterile cows, mostly functionally sterile, it can be concluded that vaginal acidity is not an important factor in sterility of cattle. In two cases only among over 30 cows studied was a pH below 7.0 found. In the remaining cases the pH fluctuated with the estrous cycle between the limits of 7.0 and 8.9 and averaged approximately pH 7.9. The pH found in the sterile cows did not differ significantly from the values found in normal cows.

The cause for the decrease in pH immediately following estrus is not known. It may be that it is caused by a flowing of the more acid cervical mucus into the vagina at this time. It also may be that this lower pH is an estrogenic effect occurring, as it does, just after the height of the follicular phase of the ovaries. It appears, however, that estrogenic injections into

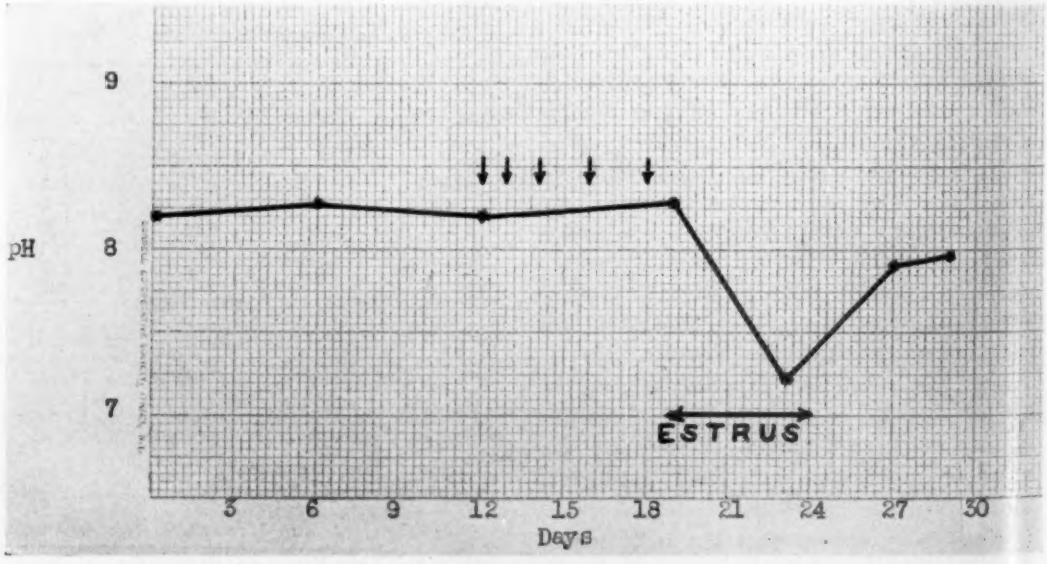


Fig. 3. pH variations of vaginal mucus of a cow following the injection of an estrogen (progynon-B) at the points indicated by the arrows. Each injection contained 10,000 R. U.

cows do not lower the pH as markedly as they do in the human or macaque females. The *modus operandi* of the estrogenic hormone in lowering the vaginal pH of the above females is not known.

That the pH of the bovine vagina is alkaline in contrast to the adult human vagina which is acid need not be surprising since other body fluids in cattle are alkaline, that is, saliva and urine. The diets of cattle have an alkaline residue while the usual human diet has an acid residue. It would be interesting to see if the vagina of the cow can be made acid by feeding a diet that has an acid residue.

The results obtained by Andrews in the mare closely parallel our results obtained in cattle. The pH level of the vaginal mucus is about the same and the cervical mucus is more acid than the vaginal mucus in both cases. Also, the pH in the mare is lowest following estrus. The low point appears to be at about two days in cattle, whereas in the mare it is at five to ten days post estrus.

The fact that the bovine cervix is more acid than the vagina raises some interesting questions in regard to the ascent of spermatozoa in the genital tract. In the human female the cervix is more alkaline than the vagina (Meaker and Glasser,[1] Kessler and Uhr,[18] Miller and Kurzrok[19]), and it has been suggested that this H-ion difference is an aid to the movement of spermatozoa, that is, spermatozoa are chemotaxic. If sperms are attracted by the more alkaline cervix in humans, would they be repulsed by the relatively acid cervix in cattle? If a general mechanism guides the ascent of spermatozoa in the genital tract of mammalian species, it would seem to be other than a pH differential, since in these two species—cattle and man—the differences are in opposition to each other.

Inasmuch as vaginal acidity was not found to be an important factor in sterility among the cattle studied, is the often-recommended sodium bicarbonate douche prior to service a waste of time? It would appear that, from the standpoint of a neutralization of the vaginal tract, it is. As mentioned previously, Wester[7] used sodium bicarbonate douches in cattle with what he considered success. His explanation was that spermatoxins of an unknown nature were removed. This phase of the problem is interesting enough to warrant further work. The douches may be beneficial by removing debris to which spermatozoa may be attracted. If this is so, there is no reason to believe that bicarbonate douches would be any better than saline douches.

The additional fact should be pointed out that spermatozoa are deposited in the vagina at a time when the pH of the vagina is still quite alkaline and that they probably leave the vagina before the time of least alkalinity.

There is another point that will be of interest in the light of the recent discussions concerning the effect of alkaline and acid vaginal douches on the sex of the offspring. If, as is stated, alkaline douching of the vaginas of animals leads to the production of a preponderance of male offspring and acid douching leads to the production of a preponderance of female offspring, should we not expect that those species having an acid vagina, as man and monkeys, should have a greater number of female offspring, or on the other hand, that animals having an alkaline vagina as cows and mares, should have a preponderance of male offspring? The secondary sex ratio of each of the above species is near equality.

SUMMARY AND CONCLUSIONS

In a series of over 30 normal and sterile cows the vaginal pH as determined by a glass electrode was found to be alkaline. With the exception of two isolated cases the pH ranged from 7.0 to 8.9 and averaged pH 7.9.

The vaginal pH varied with the estrous cycle, being high during diestrum and estrus and low during metestrum.

Doses as high as 50,000 R. U. of an

[18] Kessler, R., and Uhr, E.: Biologie und Chemismus der Scheide bei Schwangeren. Arch. Gynak., cxxix (1927), p. 844.

[19] Miller, E. G. Jr., and Kurzrok, R.: Biochemical studies of human semen. III. Factors affecting migration of sperm through the cervix. Amer. J. Obst. & Gynec., xxiv (1932), p. 19.

Enteritis in Sheep Caused by Infection with the Protozoan Parasite Globidium Gilruthi*

H. MARSH, D.V.M., and E. A. TUNNICLIFF, D.V.M.

Bozeman, Mont.

IN THE LITERATURE on the protozoa there are a number of references to a genus of intestinal parasites which are somewhat similar to the Sarcosporidia, the generic name being Globidium.

REVIEW OF LITERATURE

These reports were summarized in Wenyon's *Protozoölogy* (1926).[1] Species have been described from the horse, sheep, goat, cattle, kangaroo, wallaby, wombat and armadillo. Parasites of this type were first observed in sheep by Moussu and Marotel[2] in 1902. They were reported by Gilruth,[3] in 1910, as occurring in the abomasum of sheep, and Chatton,[4] in 1910, described the species, giving it the name *Gastrocystis gilruthi*. In 1925, Triffitt[5] found *G. gilruthi* in 127 of 138 sheep selected at random from London slaughterhouses. She stated that the parasite was not found in the intestine, but only in the abomasum. In 1931, Canham[6] noted the occurrence of *G. gilruthi* in the abomasum of sheep in Natal. No mention of this parasite has been found in the American literature, but Benjamin Schwartz, chief of the Zoölogical Division of the United States Bureau of Animal Industry, has stated in a personal communication that he has in one instance identified this organism in the abomasum of a sheep.

Apparently the life history of Globidium is not known, and there seems to be little information as to its pathogenic effect. Therefore, it may be of value to record here some observations on a severe enteritis which developed in sheep as a result of heavy infection with Globidium.

FIELD OBSERVATIONS

In April 1940, an investigation by this laboratory of a severe diarrhea in sheep on two ranches in the Yellowstone Valley established a heavy infection with Globidium as the cause of the disease. In the first instance in which this diagnosis was made, the disease occurred in a band of 1,360 ewes in the latter part of March and early April. A few cases of diarrhea were noticed about March 26, and by April 4, when the first examination was made by Dr. H. C. Roquet of Big Timber, there were estimated to be between 300 and 400 cases in the band. The number of cases developing tapered off after April 4, but the total number reached about 450, with a death loss of 40. The survivors made a very slow recovery.

SYMPTOMS AND COURSE

In the acute stage of the disease, the affected sheep developed a severe diarrhea. There was no evidence of blood in the material passed, except that after several days the straining became severe, and blood appeared in the feces in some cases as a result

*From the Montana Veterinary Research Laboratory, Montana Agricultural Experiment Station and Montana Livestock Sanitary Board coöperating. Paper No. 143, journal series, Montana State College, Agricultural Experiment Station.

[1]Wenyon, C. M.: Protozoölogy (Bailliere, Tindall and Cox, London, 1926), Vol. 1, pp. 769-773.

[2]Moussu, G., and Marotel, G.: La coccidiose du mouton et son parasite. Arch. Parasitol., vi (1902), p. 82. Cited by Wenyon.

[3]Gilruth, J. A.: Notes on a protozoon parasite found in the mucous membrane of the abomasum of a sheep. Bul. Soc. Path. Exot., iii (1910), p. 297. Cited by Wenyon.

[4]Chatton, E.: Le kyste de Gilruth dans la muqueuse stomacale des ovides. Arch. Zool. Exp. et Gén., xlv (1910), 5th Ser., Vol. 5, notes et rev., 4, pp. cxiv-cxxiv. Abstracted by Wenyon.

[5]Triffitt, Marjorie J.: Observations on *Gastrocystis gilruthi*, a parasite of sheep in Britain. Protozoöl., i (1925), pp. 7-18.

[6]Canham, A. S.: A note on the occurrence of *Globidium gilruthi* in Natal. J. So. African Vet. Med. Assn., ii (1931), pp. 45-46.

(*Continued from preceding page*)

estrogenic hormone failed to produce an acid vaginal reaction in four cows.

The mucus of the cervix is more acid than the mucus of the vagina; the former averaged pH 6.8 and the latter pH 8.5 in the four cows studied.

The flowing away of the cervical mucus at estrus may account for the decrease in vaginal pH immediately following estrus.

of a hemorrhagic condition of the rectum. The sheep were dejected, the abdomen was contracted, and they lost flesh rapidly. There was no fever. The acute enteritis and diarrhea persisted for about one week, after which the 90 per cent of the affected sheep which survived the acute attack recovered slowly. A ewe which was brought to the laboratory on April 9 had at that time a profuse diarrhea, the material passed being a thin fluid. On April 12, the feces were soft but approaching normal, and on April 15, the feces were normal. When received, this ewe was thin and the abdomen was greatly contracted. For the next 30 days there was no noticeable change in her appearance. On May 17, it was noted that she was beginning to fill out, and on May 29 she was reported as being in fair condition.

Gross Pathology

Autopsies were performed on five sheep. Two of these were on fatal cases of the disease, one from each of the two ranches involved. Two of the autopsies were on sheep that were very sick and were killed for study. One was on a sheep which was convalescent, but as yet showed no improvement in general condition.

While *Globidium gilruthi* has previously been reported as a parasite of the abomasum, in these cases the principal lesions were in the small intestine. In one case the parasites were present in the abomasum in considerable numbers, but in general there was no gross pathologic change in that organ.

In one of the fatal cases the mucosa of the entire length of the small intestine was studded with small white cysts, ranging in diameter from 200 μ to 500 μ. Some of the cysts were at the surface of the mucosa, and others were deeply embedded. The cyst wall was a thin membrane, surrounding large numbers of oval to lanceolate bodies about 10 μ in length. No motility was observed in these bodies when they were released by breaking the cyst wall. The intestinal mucosa was hyperemic, and the surface had a somewhat furry appearance.

In another autopsy on a ewe killed in the

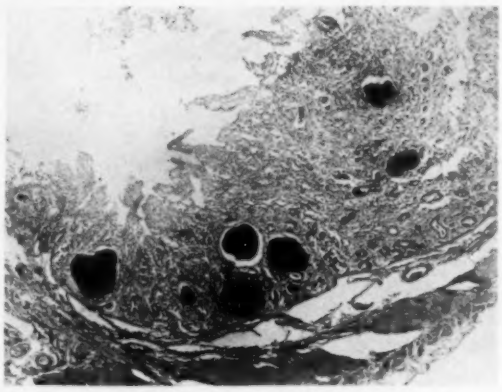


Fig. 1. Section of intestinal mucosa, showing loss of epithelium, destruction of intestinal glands, and several Globidium cysts in various stages of development (x 28).

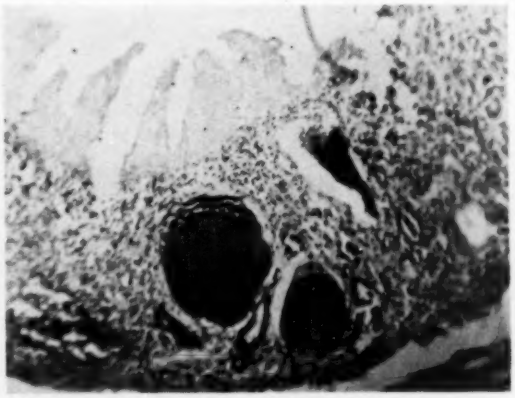


Fig. 2. Section of intestinal mucosa, showing villi denuded of epithelium and containing no-nuclear structures, lymphocytic infiltration of mucosa, and three Globidium cysts (x 85).

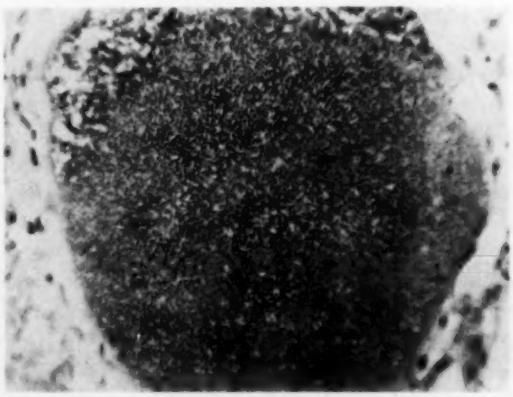


Fig. 3. Mature Globidium cyst (x 325).

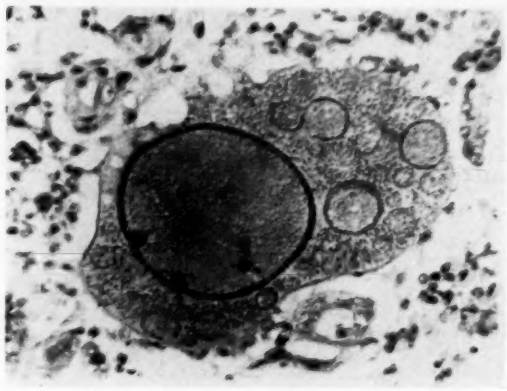


Fig. 4. Immature Globidium cyst (x 325).

acute stage of the disease, there were a few Globidium cysts in the duodenum, the number increasing as the intestine was followed back, until the cysts were less than 1 mm. apart in the mucosa of the posterior portion of the small intestine.

The gross pathology of a convalescent case was studied in a ewe which was brought to the laboratory in the acute stage, and was killed 30 days later, when recovery had started. The mucosa of the small intestine did not show any marked gross pathology. There was capillary injection, which showed as fine red streaks throughout the intestinal mucosa. There were only a few of the Globidium cysts scattered through the mucosa.

None of the autopsies showed helminth parasites in sufficient numbers to be factors in causing the disease observed. Coccidial oöcysts were observed in scrapings from the mucosa of the ileum and rectum in several instances, and numerous oöcysts were present in fecal samples from two sick sheep which were brought to the laboratory. As coccidia are constantly present in the intestines of sheep in this region, it is not thought that the coccidia are primarily responsible for the enteritis described here.

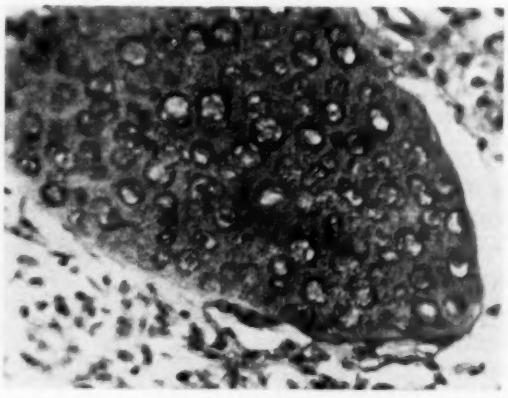


Fig. 5. Immature Globidium cyst (x 325).

HISTOPATHOLOGY

Sections of the intestine of a sheep which died in the acute stage of the disease showed extensive histopathologic changes (figs. 1 and 2). The villi of the mucosa were completely denuded of epithelium, and many of the villi were entirely destroyed. Nearly all the epithelium of the necks of the glands of Lieberkühn was destroyed. The epithelium of the fundus of the glands was intact in many instances. The outer portion of the mucosa, in which the tubular portion of the glands of Lieberkühn normally occur, contained a mass of infiltrating lymphocytes and monocytes, with some nuclei of the degenerated epithelial cells of the glands.

Embedded in the mucosa at varying depths were large numbers of Globidium cysts, varying in diameter from 75 μ to 250 μ. The majority of these bodies consisted of thin-walled, roughly spherical cysts (fig. 3), containing masses of lanceolate spores about 10 μ in length, rounded at one end and pointed at the other, with a small nucleus near the rounded end. In part of the cysts the

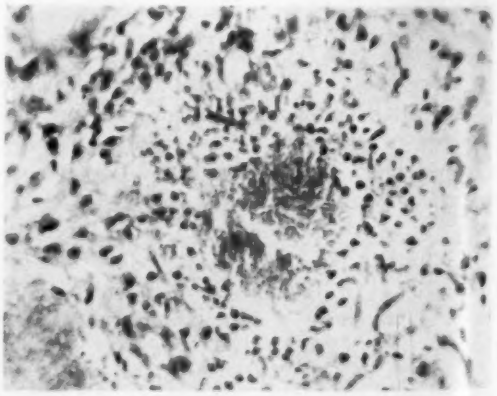


Fig. 6. Group of Globidium spores, showing no cyst wall, surrounded by monocytes and lymphocytes (x 325).

spores were short and approached the spherical form, apparently an early stage in the development of the lanceolate spores.

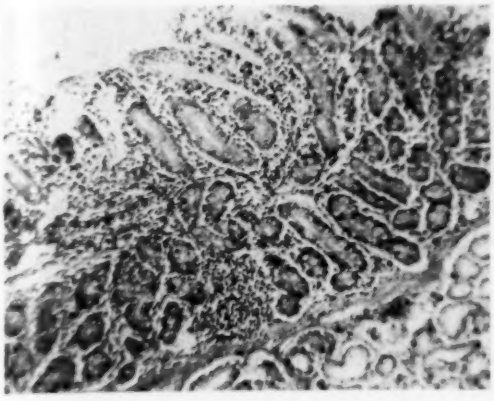


Fig. 7. Section of mucosa of duodenum of convalescent sheep, showing loss of epithelium of villi, normal glands of Lieberkühn and Brunner, and lymphocytic infiltration (x 85).

In a small percentage of the cysts, usually those deeply embedded, there were within

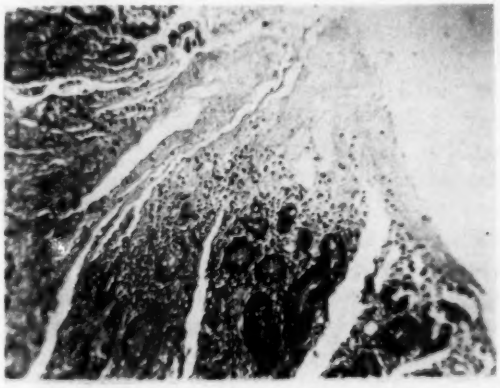


Fig. 8. Section of mucosa of ileum of convalescent case, showing lack of epithelium on surface, cellular infiltration, and regenerating intestinal glands (x 85).

the cyst membrane many spherical bodies of varying size, the surface of which was studded thickly with small, compact, nuclear bodies, which later develop into the lanceolate spores (figs. 4 and 5). At several points there were small groups of the spores free in the tissue of the mucosa (fig. 6).

Sections of the abomasum, duodenum, and ileum of the convalescent ewe were examined. The abomasum sections showed no abnormality except that nearly all the epithelial cells of the tubular portion of the glands were distended with mucus. No Globidium forms were seen in the sections. The sections of the duodenum (fig. 7) showed loss of epithelium from the villi, but the supporting tissue of the villi appeared normal. The glands of Lieberkühn and Brunner's glands appeared normal. There was extensive infiltration of lymphocytes into the mucosa. No Globidium forms were seen in the sections. The sections of the ileum (fig. 8) showed that the surface of the mucosa was devoid of epithelium. The glands of Lieberkühn appeared to be in the process of regeneration. In some sections these glands appeared practically normal, except that nearly all the cells were distended with mucus. In other sections the epithelium of the glands showed the appearance of rapid growth, with the cells crowding each other in somewhat irregular formation. The tissue surrounding the glands was infiltrated with large numbers of lymphocytes, plasma cells, monocytes and eosinophiles. Globidium was not found in the sections.

Summary

In this report there has been described an enteritis in sheep, accompanied by severe diarrhea, and associated with a massive infection of the intestinal mucosa with Globidium. The organism observed apparently corresponds to the species *Globidium gilruthi* in morphology, although that species has hitherto been reported as occurring only in the abomasum.

Results Obtained by Long-Continued Cultivation of Brucella Abortus in Living Chick Embryos*

H. J. METZGER, D.V.M., and FREIDA R. STOKES, M.S.
New Brunswick, N. J.

IN A PREVIOUS paper[1] it was reported that eleven egg-to-egg transfers of a virulent strain C. G., of *Brucella abortus*, and of United States Bureau of Animal Industry strain 19 of the same organism had caused no changes in the cultural characteristics, virulence or antigenic properties of either strain. The method of making these transfers was described in the aforementioned paper. This study was continued in order to determine the effect on these two strains of growth over a longer period on living chick-embryo tissue.

As the work progressed, strain C. G. continued to require carbon dioxide for good growth on artificial media up to the 39th transfer. Cultures made from this transfer and from all subsequent transfers have all grown readily without the addition of carbon dioxide. Three successive guinea pig passages of one of these cultures which grew aerobically have not changed this cultural characteristic. No changes whatever were noted in the cultural characteristics of strain 19 up to the 55th transfer. It continues to grow without the addition of carbon dioxide. No rough colonies were observed in cultures from any tranfers of either strain C. G. or strain 19. Antigens made from the 27th, 40th and 54th transfers of both strains have checked exactly with the stock antigen used in this laboratory and with the antigens made from the two original strains.

At different times during the work, guinea pig inoculations were made in order to determine if the virulence of these two strains had changed. Suspensions from the 27th transfers, C. G. (27) and 19 (27) were inoculated intraperitoneally into groups of eight guinea pigs each. Each pig received approximately 800,000 live organisms. These pigs were killed six weeks after inoculation. The blood titers and postmortem lesions exhibited by the animals inoculated with these two strains compared favorably with those obtained when the original strains were used for the inoculation of guinea pigs. This would indicate either that the 27 transfers on living chick-embryo tissues had not changed the virulence of either strain or that the change in virulence had been so slight that the methods used did not detect it.

Cultures from the 48th transfer were compared with the original cultures by guinea pig inoculation. In this work strains C. G., C. G. (48), 19, and 19 (48) were inoculated intraperitoneally into groups of four guinea pigs each. Approximately 100,000 live organisms were inoculated into each pig. The two original strains used in this work had been held in the laboratory for over a year by making monthly transfers on potato agar. On postmortem examination at the sixth week, the guinea pigs inoculated with the original C. G. strain showed enlarged and roughened spleens, small necrotic foci on the surface of the liver and in some cases atrophied testes. With the exception of one guinea pig inoculated with the original strain 19 the pigs in the other three groups did not show any macroscopic lesions. This pig showed an enlarged and roughened spleen.

Two more successive guinea pig passages of this C. G. (48) variant which was recovered from the above inoculation have shown no evidence of increased virulence insofar as macroscopic lesions or blood titers are concerned.

To study further the effect of the long-

*Journal series paper of the New Jersey Agricultural Experiment Station, Department of Dairy Husbandry. Presented before the Section on Research at the 77th annual meeting of the American Veterinary Medical Association, Washington, D. C., August 26-30, 1940.

[1]Metzger, H. J., and Stokes, Freida R.: J.A.V.M.A., xcvi (1940), pp. 369-371.

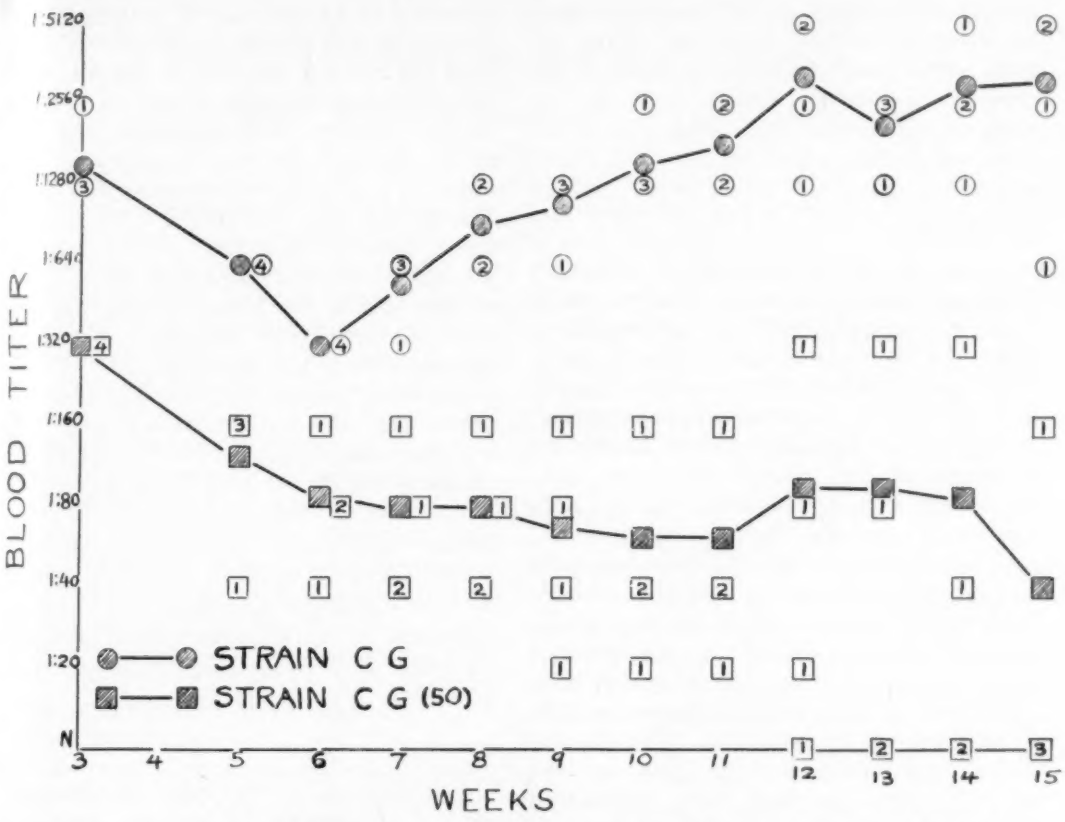


Fig. 1. Average whole blood titers of guinea pigs inoculated with Br. abortus C. G. and C. G. (50). The weekly distribution of titers is represented by hollow squares and circles. Each square and circle is labeled to indicate the number of guinea pigs reacting in that titer.

continued growth of strain C. G. in living chick embryos, the original strain was compared with the 50th transfer of this strain, C. G. (50), with respect to its ability to produce agglutinins in the bodies of guinea pigs. Two groups of four guinea pigs each were inoculated intraperitoneally, one group receiving the original strain and the other group, strain C. G. (50). Each pig received approximately 1.5 billion live organisms as determined by a plate count.

Beginning with the third week, the blood titer was determined on each pig by a method which was reported by Hagan.[2] One-tenth cc. of blood is removed from the ear of the guinea pig and added to 0.9 cc. of a citrated physiological saline. The sample is left in the refrigerator overnight after which time as much as possible of the clear supernatant fluid is removed to another tube. By adding antigen equal to the amount of supernatant fluid in the second tube, a 1:20 dilution is obtained. Other dilutions are made from this tube. This method gives the titer on the whole blood instead of the serum. Such titers are approximately one-half that of the serum.

Figure 1 gives a comparison of the whole blood titers of the two groups of guinea pigs from the third to the 15th week. It is apparent that while there are some individual differences within each group, the blood titers of all of the pigs inoculated with the original strain C. G. were definitely at all times at a higher level than the blood titer of those inoculated with strain C. G. (50). It should be added that all of the guinea pigs inoculated with strain C. G.

[2] Hagan, W. A.: J. Exp. Med., xxxvi (1922), pp. 711-725.

developed lameness due to an involvement of either the carpal or tarsal joints, or both. This was first noted in three of the pigs on the eighth week and in the remaining pig on the ninth week. No lameness was noted in any of the C. G. (50) group.

DISCUSSION

The above results indicate that the long-continued cultivation of *Br. abortus*, strain C. G., in living chick-embryo tissues has reduced the virulence of this strain as far as its ability to produce macroscopic lesions and high blood titers in guinea pigs is concerned. It has also changed the carbon dioxide requirement of this strain. Somewhat similar results were obtained by Salle and Robinson,[3] by growing type II and type III pneumococcus and type II meningococcus on minced chick-embryo tissue suspended in Tyrode's solution. They found that this medium rapidly reduced the virulence of these organisms. They suggested that their results were "due to some substance or substances present in embryonic chick tissue." Their results differed from those obtained in this work in that their attenuated strains were rough. The *Br. abortus* cultures from all egg-to-egg transfers have been smooth.

It is of interest to compare the results obtained by serial egg-to-egg transfers of a virulent strain of *Br. abortus* with the results obtained by MacEwen[4] by serial transfers in guinea pigs of a strain of *Br. abortus* of low virulence. MacEwen measured the toxicity for mice of the 6th, 20th, 30th and 40th passage variant of a strain of low virulence and concluded that with serial passages through guinea pigs there occurred a progressive increase of toxicity. The results obtained by the inoculation of these same variants into guinea pigs and pregnant cattle indicated that the virulence of this strain for these animals had also been progressively increased by serial passage through guinea pigs.

MacEwen's work with guinea pigs and the work with the incubating eggs both indicate that it is possible to change the virulence of *Br. abortus* by growing it in living tissues. Both pieces of work would indicate that the virulence of this organism is not likely to undergo a sudden change. In both experiments a change in virulence, sufficient to be of importance clinically, occurred only after long-continued rapid transfers on the medium used.

SUMMARY

Repeated egg-to-egg transfers have made certain changes in a virulent strain of *Brucella abortus*, strain C. G. Since the 39th transfer this organism has been growing on artificial media without the addition of carbon dioxide. A comparison of the 48th and 50th transfers of this strain with the original C. G. indicates that the organism has lost much of its virulence and of its ability to stimulate the production of antibodies in guinea pigs.

[3]Salle, A. J., and Robinson, J. L.: J. Immunol., xxxvi (1939), pp. 385-394.

[4]MacEwen, A. D.: Vet. Rec., lii (1940), pp. 97-106.

Bacterial Dissociation in Brucella Abortus*

C. K. MINGLE, D.V.M., M.Sc., and C. A. MANTHEI, D.V.M.
Beltsville, Md.

THE GENERAL problem of dissociative variation has been recognized for the past two or three decades. However, it is only within recent years that concerted effort on the part of interested workers has brought a better understanding of this phenomenon.

In much of the earlier data relating to scattered instances in which dissociation has been noted, the study of the variations themselves was seldom the primary object of investigation. This resulted in the accumulation of considerable information on the subject which was in many instances inconclusive and often misleading. The speculations of various investigators as to the exact nature and significance of the many changes involved naturally led to much contention and disagreement.

The subject assumed added importance with the realization that a possible application of the principles of bacterial instability might be made to certain notable variations in the clinical manifestations of many infectious diseases.

Through the work of several competent investigators, it has been established that dissociation in the Brucella group of organisms is comparable with that exhibited by most species. Among the first to observe the so-called R or rough type in *Brucella abortus* was Henry,[1] who described an opaque granular colony, suspensions of which were characterized by spontaneous agglutination. Further studies reported by Traum and Henry,[2] as well as Plastridge and McAlpine,[3] definitely established the fact that dissociative changes are common in most Brucella cultures.

Henry,[4] in 1933, pointed out that the probable course of dissociation in the genus Brucella is S (smooth) through an intermediate form to R (rough), and reversion of the R to the primary S type is very improbable. It is now generally recognized that these changes may take place either as a spontaneous process or as the result of artificially controlled procedure.

The increasing use of *Br. abortus* strain 19 in the production of vaccine has emphasized the value of a thorough knowledge of cellular and colonial morphology correlated with immunologic properties. The importance of determining the nature and significance of dissociative forms occurring in such a culture is readily apparent. Moreover, it is obvious that only through an understanding of these manifestations can any rational procedure for maintaining a desirable stability be successfully employed.

Considerable data relative to a logical classification of the commonly observed culture phases are available. Therefore, the material to be presented in this paper will include some observations which are confirmatory of reported work, as well as new findings.

The use of obliquely transmitted light has been effectively employed in these studies for the detection of colonial variations. The apparatus is a modification of similar equipment described by Henry[4] in 1933. An ordinary binocular dissecting microscope with a lens combination giving a magnification of about 15 diameters is suitable for observing colonial types as well as selective subculturing. A 6-volt frosted lamp, mounted in an adjustable focusing reflector, so arranged as to allow the beam to penetrate the Petri dish at an upward angle of approximately 45 degrees, serves

*From the Animal Disease Station of the Bureau of Animal Industry, U. S. Department of Agriculture. Presented before the Section on Research at the 77th annual meeting of the American Veterinary Medical Association, Washington, D. C., August 26-30, 1940.

[1]Henry, B. S.: Spontaneous and forced dissociation of *Brucella abortus* Bang. Proc. Soc. Exp. Biol. & Med., xxvi (1928), p. 101.

[2]Traum, J., and Henry, B. S.: Discrepancies of the agglutination test in *Brucella abortus* infection in cattle. Cornell Vet., xix (1929), p. 105.

[3]Plastridge, W. N., and McAlpine, J. G.: Microbic dissociation in the abortus-melitensis group. J. Inf. Dis., xlvi (1930), p. 315.

[4]Henry, B. S.: Dissociation in the genus Brucella. J. Inf. Dis., lii (1933), p. 399.

as adequate lighting. With this arrangement it is possible to demonstrate colony variations which are not detectable under the usual vertical lighting arrangement.

Inasmuch as the practical significance of bacterial instability would seem to lie primarily in its manifestations under usual laboratory procedure, the methods and materials employed have conformed with these conditions insofar as possible.

A broad classification of the colonial forms observed in strain 19 and commonly associated with most Brucella cultures consists of three main groups: the S (smooth), I (intermediate) and R (rough) types.

The S type conforms to the general concept of smooth forms described for most bacterial species. The colony is characteristically small, bluish and translucent with regular margins and a smooth, glistening surface. The individual cells are uniformly short rods, 0.5 to 1.5 μ in length, occurring singly in stained preparations. As would be expected, stable suspensions are readily prepared in normal physiological saline. While the smooth type is generally associated with virulent cultures, pathogenicity is not necessarily a distinctive attribute of these forms. This is evident in the fact that repeated selective subcultures of typical S forms fail to increase materially the virulence of strain 19. Antigenically, the S organisms are similar to those associated with virulent infections.

The R type, descriptive of terminal dissociative forms, presents some of the colonial characteristics common to this dissociant in other bacterial groups. However, the irregularity of margins usually observed is less pronounced in Brucella cultures. In common with R colonies of other genera, those of Brucella are markedly opaque and present a dull, granular appearance. The convex surface, characteristic of smooth colonies, is considerably less marked in the rough type. This restricted elevation becomes progressively accentuated with increasing age of the culture. The individual cells, occurring singly, are usually somewhat larger than the S type, although an occasional long, slender rod may be observed. Instability of bacterial suspensions prepared in .85 per cent saline is quite characteristic of the R types. Loss of virulence, as well as altered antigenic and physiological activity, is also observed.

Progressive variations in colonial appearance, occurring as a gradual process in the S to R conversion, tend to complicate the classification of so-called I or intermediate forms. Furthermore, any satisfactory classification must take into consideration the fact that antigenic and physiological changes do not necessarily parallel detectable variations in colonial morphology.

The intermediate forms are, as a rule, somewhat larger than either their S or R prototypes, although this variation in colony size is less pronounced in a comparison of the I and R than between the I and S forms. Most of the I types exhibit a moderate degree of granularity while otherwise retaining the colonial and antigenic qualities peculiar to the smooth colonies. As the I to R conversion progresses toward the R stage, associated changes in antigenicity are usually noted.

In view of the recognized continuous nature of the entire process of dissociation, culture phases should and do exist which are intermediate between the S and I as well as the I and R forms. These are usually quite unstable and prove difficult to propagate in pure culture.

Another variant possessing characteristics distinctly different from the usual types has been recently observed in strain 19. It was first noted under rather peculiar circumstances in connection with routine tests conducted on a vaccine sample submitted for examination. This product had been prepared at an educational institution for use in experimental vaccination studies. The following history accompanied the sample. Thirty days following the vaccination of 21 calves, blood-agglutination tests showed 15 of the vaccinated animals to be completely negative, while the remaining six exhibited only a negligible response which promptly subsided. Revaccination of the same group three months later induced slight titers in ten of the inoculated animals, all of which were again negative at the end of 30 days. Cultural examination of this product in our laboratory revealed a relatively high percentage of dissociative

forms common to Brucella cultures. In addition to these types, an abundance of unusual colonies characterized by slightly irregular margins and uniformly roughened or contoured surfaces was observed. These colonies exhibited a granularity comparable with that observed in the usual intermediate forms. When examined macroscopically, they were indistinguishable from typical Brucella. In comparison with the R type, they were less friable and appeared to be somewhat viscous.

Original picked cultures produced recognizable Brucella types as well as the parent forms. Repeated selective subcultures eventually established the variant to a point where is was possible to cultivate uniform colony types. On preliminary examination, this culture, designated strain C-W, exhibited distinct differences in its antigenic relationship to the typical S types. The possibility that we might be dealing with a dissociant of Brucella prompted an examination of all available out-dated vaccine for the presence of comparable types. From two different lots, each over six months old, a few colonial forms of similar character were detected. Culturally and morphologically they appeared similar to the C-W strain. The recovery of corresponding colony types from different sources, each in connection with identical products, encouraged a comprehensive investigation of their relationship to one another as well as to smooth Brucella forms.

The four Brucella strains employed in this comparative study are identified, respectively, as C-W (original variant), B-W (variant recovered from out-dated vaccine), 214 (virulent smooth culture), and 19 (stock vaccine culture).

Studies of Dissociant W Strains

Colonial Morphology

The W colonies are characterized by a contoured or wrinkled surface, the margins of which present only slight irregularities. Mallmann and Gallo[5] state that the surface of the R colony is dull in appearance, flat, spreading, and occasionally wrinkled. The colonies exhibit a granularity comparable with that observed in the usual intermediate forms. In general, they are less friable than the R type and appear somewhat viscous.

Cell Morphology and Staining Reactions

In stained preparations, the organisms exhibit a marked tendency for chain formation where appropriate care, exercised in the preparation of slides, has prevented their dissolution. The individual cellular elements are uniformly larger than those of the S colonies. The average size is 2 to 3 ft. long by 0.5 to 1 μ wide. An increased size of the R types was noted by Marshall and Jared[6] in 1931. The reaction to Gram's stain is typical of the usual S forms.

Biochemic Reactions

Biochemic tests, including carbohydrate fermentation, dye tolerance and hydrogen sulfide production, were carried out with the W strains in comparison with parallel tests of S cultures.

Carbohydrate Fermentation.—The fermentative powers of the variant forms were tested with the following carbohydrates: levulose, galactose, glucose, mannitol, maltose, arabinose, rhamnose, sorbitol, xylose, dulcitol, lactose, sucrose, raffinose, salicin, dextrin, inulin, esculin and trehalose.

The test broth employed was a sugar-free product to which the various carbohydrates and indicator solution (Andrade's) were added in 1.0 and 0.5 per cent amounts, respectively.

Observations at 24-hour intervals over a period of 7 days of incubation at 37° C. failed to reveal any appreciable acid production by either of the test cultures or the two S type controls. Henry[4] made similar observations and pointed out that the characteristic inactivity exhibited by *Br. abor-*

[5] Mallmann, W. L., and Gallo, F.: Studies on the dissociation of the Brucella group. J. Agr. Res., xlvi (1933), p. 272.

[6] Marshall, M. S., and Jared, Dorothy: Microbic dissociation in the Brucella group. J. Inf. Dis., xlix (1931), p. 323.

TABLE I—Bacteriostatic Action of Dyes on S and W Cultures of Brucella

Culture	Type	Relative Growth of Indicated Dye and Control Plates			
		Control	Thionin	Basic Fuchsin	Methyl Violet
214 (bovine)	S	++++	—	+++	+++
19 (bovine)	S	++++	—	++++	++++
xxx (suis)	S	+++++	++++	—	—
2978 (melitensis)	S	+++	++	++	++
C-W	Variant (W)	++++	++++	++++	++++
B-W	Variant (W)	++++	++++	++++	++++

Thionin 1:20,000—National Analin Co.—NT-1.
Basic fuchsin 1:30,000—National Analin Co.—NC-8.
Methyl violet 1:60,000—National Analin Co.—NF-13.

tus in relation to carbohydrate fermentation was not altered in the several variants studied.

Dye Tolerance.—To determine possible variations in the tolerance to dyes, plates containing thionin, basic fuchsin, and methyl violet, respectively, were prepared as described by Huddleson[7] for the differentiation of Brucella species.

Heavy, 48-hour saline suspensions of uniform density were prepared with the C-W and B-W variants as well as strains 19 and 214. These were employed in identical amounts to seed two sets each of the three dye plates. Observations were made after 72 hours of incubation at 37° C. The results are recorded in table I.

It was observed that both variant test cultures grew equally well on all of the plates, indicating a marked tolerance for the dyes employed. The control cultures, on the other hand, were inhibited by the thionin but not by the basic fuchsin or methyl violet.

These results are in accord with those of Mallmann and Gallo[5] and Marshall and Jared[6] who observed a similar tolerance for the R forms studied. It would appear that these variant forms can not be typed by a procedure based on the inhibiting action of the dyes employed.

Production of Hydrogen Sulfide.—Huddleson's[7] method of typing Brucella cultures by the degree of hydrogen sulfide production was employed in comparative tests.

Lead-acetate paper strips were held in position by the cotton plugs in heavily seeded agar slants incubated at 37° for four days. The paper strips were replaced at 24-hour intervals.

The results of repeated tests were inconclusive and in several instances considerable irregularity in the production of hy-

[7]Huddleson, I. F.: The differentiation of the species of the genus Brucella. Michigan Agr. Exp. Sta. Tech. Bul. 100 (1929).

TABLE II—Cross-Agglutination Reactions

Serum	Antigen				
	214	19	C-W	B-W	C-S
	Maximum Dilution Showing Complete Agglutination				
214	5,120	5,120	0	0	5,120
19	2,560	2,560	50	50	2,560
C-W	0	0	2,560	2,560	0
B-W	0	0	2,560	2,560	0

214 (virulent S), 19 (stock vaccine culture), C-S (reverted C-W), C-W (variant W), B-W (variant W).

drogen sulfide by the variant forms could not be correlated with known factors. Similar irregularities were observed by Marshall and Jared[6] in their study of variant Brucella types.

The suitability of the medium employed in this procedure was indicated by the fact that the reactions with the known S forms were sufficiently clear cut to permit an accurate classification of the respective control cultures.

Serologic Studies

Serologic studies of the variant W forms were made for a comparison of their antigenic relationship to the smooth 19 and 214 cultures.

Immune rabbit serum was prepared against each of the two variant cultures (C-W and B-W) as well as the two S strains (19 and 214)). Care was exercised in the preparation of subcultures for inoculation purposes to insure a constant uniformity of type in the antigens employed as immunizing agents. Each rabbit received four biweekly injections of respective cultures. These injections, each standardized to approximately 10^9 organisms per cubic centimeter, were administered intraperitoneally. Seven days after the final injections, the rabbits were bled from the heart and the agglutination titer of the serums for the homologous and heterologous antigens determined.

The results of these agglutinations are presented in table II. It will be observed that complete agglutination occurred with strains and homologous antiserums in approximate titers of 1:2,560. Heterologous smooth strains agglutinated to maximum titers with each of the S antiserums. Likewise, heterologous variant cultures agglutinated to titer with the W antiserums. However, no agglutination of the variant antigens by either of the smooth antiserums was noted. Some relationship appears to

TABLE III—Results of Agglutinin Absorption with Indicated Antigens

Anti-Serums	Absorbing Culture	Agglutination Titer Before and After Absorption								
		20	40	80	160	320	640	1280	2560	5120
214	——	+	+	+	+	+	+	+	+	0
	214	0	0	0	0	0	0	0	0	0
	19	0	0	0	0	0	0	0	0	0
	C-5	0	0	0	0	0	0	0	0	0
	C-W	+	+	+	+	+	+	+	+	0
	B-W	+	+	+	+	+	+	+	I	0
19	——	+	+	+	+	+	+	+	+	0
	19	0	0	0	0	0	0	0	0	0
	214	0	0	0	0	0	0	0	0	0
	C-S	0	0	0	0	0	0	0	0	0
	C-W	+	+	+	+	+	+	0	0	0
	B-W	+	+	+	+	+	+	I	0	0
C-W	——	+	+	+	+	+	+	+	+	0
	C-W	0	0	0	0	0	0	0	0	0
	19	+	+	+	+	+	+	0	0	0
	214	+	+	+	+	+	+	I	0	0
	C-S	+	+	+	+	+	+	0	0	0
	B-W	0	0	0	0	0	0	0	0	0
B-W	——	+	+	+	+	+	+	+	+	0
	B-W	0	0	0	0	0	0	0	0	0
	C-W	0	0	0	0	0	0	0	0	0
	19	+	+	+	+	+	+	+	0	0
	214	+	+	+	+	+	+	+	+	0
	C-S	+	+	+	+	+	+	+	0	0

214 (virulent S), 19 (stock vaccine culture), C-S (reverted C-W), C-W (variant W), B-W (variant W).
+ = Complete agglutination.
I = Incomplete agglutination.
0 = No agglutination.

TABLE IV—Reversion of C-W Dissociant by Animal Inoculation

Guinea Pig No.	Material Injected	Spleen			Blood Titer
		Microscopic Lesions	Isolations	Culture Type	
74588	19-S	No lesions	Pos.	Smooth	1:50
74589	19-S	No lesions	Neg.	—	Neg.
74590	19-S	Sl. nodules	Pos.	Smooth	1:100
74591	19-S	Sl. nodules	Pos.	Smooth	1:200
74592	19-S	Sl. nodules	Pos.	Smooth	1:100
74593	19-S	No lesions	Neg.	—	1:100
74594	C-W	No lesions	Neg.	—	Neg.
74595	C-W	No lesions	Pos.	Smooth	Neg.
74596	C-W	No lesions	Neg.	—	Neg.
74597	C-W	No lesions	Neg.	—	Neg.
74598	C-W	No lesions	Pos.	Smooth	1:25
74599	C-W	No lesions	Pos.	Smooth	Neg.

exist between the stock 19 and both variant strains as evidenced by the complete agglutination in low dilutions of the 19 antigen with B-W and C-W antiserums. Otherwise the antigenic relationship between the S and variant forms appears to be negligible.

Agglutinin-absorption tests were conducted with the same four antiserums to evaluate further the possible serologic relationships.

Preliminary titrations were made to determine the optimum amount of organisms suitable for absorption. Krumwiede, Cooper, and Provost[8] have shown that there is a possibility of overabsorption, especially if closely related groups are to be studied.

The serums were diluted 1:10 in normal physiological saline before absorption to furnish sufficient electrolyte for dispersion of the organisms throughout the mixture. Based on the preliminary titrations, necessary adjustments in the titers of the serums were made so that it was possible to standardize the amounts of the various absorbing antigens employed throughout.

Two 2-hour absorptions were carried out at 37° C. Centrifuge tubes, closed with rubber stoppers, were used as containers for the absorbing organisms and diluted serum. Thorough shaking of the serum-antigen mixtures was regularly practiced at ten-minute intervals throughout the incubation periods. Following the final absorptions, the cells were centrifuged out and the absorbed serum removed for final agglutination tests.

The results presented in table III further indicate distinct antigenic differences between the W and S forms. The culture designated C-S in this table is a reverted W form derived from the C-W culture. It is interesting to note the close antigenic relationship existing between this form and strain 19. The consistency with which reduced titers are observed in absorptions of C-W and B-W antiserums with 19 antigen, as well as 19 antiserum absorbed with C-W and B-W antigens, indicates the presence of residual antigen common to all three. On the other hand, the absence of absorbing capacity of 214 antigen for either the C-W or B-W antigens is significant. The anticipated identical character of the C-W and B-W forms appears well established.

The distinct antigenic character of these dissociants is in accord with observations made by Huddleson[9] relative to the loss of specific agglutinability and agglutinin absorption capacity by the para varieties of both the abortus and suis species.

Reversion of W Forms

Several methods have been suggested by different investigators as suitable procedures for inducing the R to S reversion and have been employed with varying de-

[8]Krumwiede, C., Cooper, G., and Provost, D. J.: Agglutinin absorption. J. Immunol., x (1925), p. 55.

[9]Huddleson, I. F.: The relation of para-abortus-melitensis to abortus-melitensis. J.A.V.M.A., lxxvii (1929), p. 940.

grees of success in several bacterial groups including Brucella.

Soule,[10] working with *Bacillus subtilis*, observed that growth in 10 per cent R antiserum broth is an effective method for bringing about this change. The ease with which these reversions may be accomplished

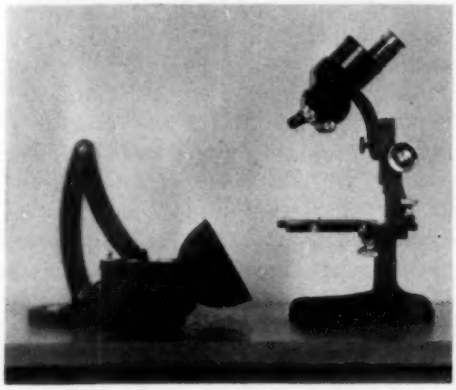


Fig. 1. Suitable lighting arrangement for the detection of colonial variations in Br. abortus cultures.

in any case probably depends to some extent upon the stage of dissociation attained. Thus, as the variant form becomes more firmly stabilized a corresponding reluctance for reversion to the S prototype is observed. Mallmann[11] demonstrated this fact in connection with studies in the Salmonella group.

An extensive study of the feasibility of various procedures for inducing the R to S change, in the variants under discussion, has not been completed at the present time. However, reversions have been successfully accomplished in several animal-passage experiments. This procedure appears to offer a fairly reliable means of bringing about this change.

Original reversion of the C-W and, later, of the B-W cultures was effected by single simultaneous inoculations administered intraperitoneally and subcutaneously.

Bacterial suspensions adjusted to a cell concentration of 10 x 10^9 organisms per cubic centimeter were prepared from 48-hour growths of the C-W variant and the smooth 19 cultures. Two groups, consisting of six guinea pigs each, were injected with the respective cultures. The individual pigs in each group received identical injections in amounts of 0.5 cc. subcutaneously and 1 cc. intraperitoneally.

All blood serums were shown to be negative for S Brucella agglutinins prior to inoculations.

The results tabulated in table IV were obtained on the autopsied pigs four weeks subsequent to injection. The abdominal and thoracic viscera were examined in each case for the presence of macroscopic lesions and all spleens cultured on potato-agar medium.

Reversion to the smooth form occurred in three of the six variant-injected pigs. In no instance was it possible to recover the

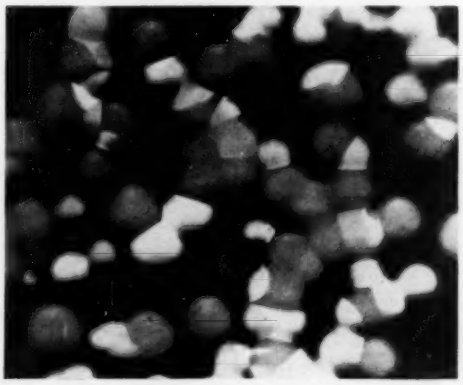


Fig. 2. Photomicrograph of extensive colony variations in Br. abortus; 96-hour culture (x 11).

original variant from the spleen, liver or testicles of any of the animals.

Absence of S agglutinins in the blood of two of the pigs from which reverted S types were recovered may be explained by the small number of organisms present. Differences in the agglutinogenic response elicited in the two groups bear out the specific antigenic nature of the variants demonstrated in the serologic studies. Further evidence of the altered character of the

[10]Soule, M. H.: Microbic dissociation in *B. subtilis*. J. Bact., xiii (1927), p. 41.

[11]Mallmann, W. L.: The dissociation of *Salmonella pullorum* and related species. Michigan Agr. Exp. Sta. Tech. Bul. 122 (1932).

variants from the standpoint of virulence is reflected in the complete absence of visible lesions in the six W-injected pigs as

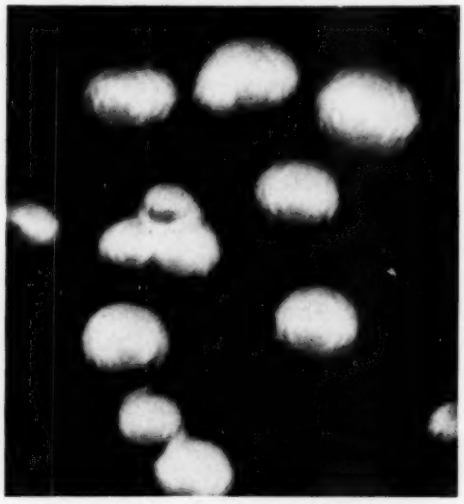


Fig. 3. Photomicrograph of variant W colonies observed under obliquely transmitted light; 96-hour culture (x 20).

compared with the presence of slight spleen lesions in three of the S-inoculated group. This reversion procedure has been duplicated with the same culture as well as the

Fig. 4. Photomicrograph of cellular elements making up variant W colonies (x 1,440).

B-W strain and results in all cases have been essentially the same as those tabulated in table IV.

A detailed study of the reverted cultures from the standpoint of cultural, biochemic and serologic characteristics has shown them to be indistinguishable from smooth strain 19.

DISCUSSION

The phenomenon of bacterial dissociation in the Brucella group is characterized by a series of gradual progressive changes occurring in the course of the S to R conversion. Many intermediate forms existing between these extreme types are for the most part unstable and tend to integrate to such an extent that their differentiation and study becomes a critical procedure. Such determinations are further complicated by the fact that antigenic and physiological changes do not necessarily parallel detectable variations in cultural characteristics.

The effect of environmental conditions on the rapidity with which observable changes occur may be considerably modified by the inherent stability of the individual strain itself. This factor is exemplified in strain 19, which, under suitable conditions, maintains a remarkable constancy of character.

The stability of most Brucella strains propagated on plain potato agar emphasizes the value of this medium for maintaining desirable culture types. However, the addition of enrichment materials, particularly serum, tends to accelerate the progress of dissociation.

Although an attempt has been made by various investigators to classify several of the unstable intermediate forms, the practical value of such procedure is questionable. It would appear that the existing confusion could be minimized and more rapid progress made in determining the nature and significance of the dissociative phenomenon by a concentration of study on the stabilized types. With a complete understanding of these forms, the unstable intermediate types may be more readily classified on the basis of their predictable characteristics.

The practical significance of bacterial instability accompanied by changes in the physical properties of bacterial antigens employed as diagnostic or prophylactic

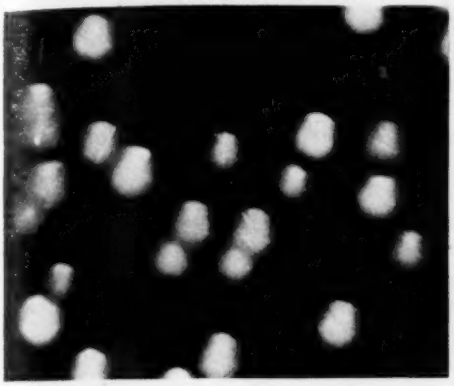


Fig. 5. Photomicrograph of reverted W colony forms, presenting most of the characteristics associated with typical S types; 96-hour culture (x 11).

agents is readily apparent. Spontaneous agglutination, characteristic of the advanced stages of dissociation, may well be a potent factor in modifying the accuracy of agglutination tests when such antigens are employed. Furthermore, the recognized variations in physiological and antigenic properties associated with certain dissociative changes which may occur in strain 19 would undoubtedly influence its value as an immunizing agent.

Routine selective subcultures of desirable types, together with periodic evaluation of their immunologic properties, must be carried out if a vaccinal product of maximum potency is to be maintained.

The unusual dissociative W forms observed in aged *Br. abortus* vaccine have been tentatively classified as intermediate types only slightly removed from the typical R forms. However, certain marked differences do exist as seen in the characteristically contoured colony surface and a pronounced tendency for chain formation of the component cells. The antigenically distinct character of this dissociant has been established by cross agglutination and agglutinin-absorption tests. Progressively difficult reversions of the W form to its S prototype have accompanied the increased stability resulting from repeated selective subcultures. The enhanced fixity of character of the W form probably accompanies a gradual elimination of residual S factors.

A study of the immunologic properties of this variant, as well as continued investigation along general lines of dissociation in the Brucella group, is in progress.

Summary

1) The results of preliminary studies of the phenomenon of bacterial dissociation in *Brucella abortus*, with special reference to strain 19, are presented.

2) A broad classification of the dissociative colonial forms observed in strain 19 comprises three main groups: the S (smooth), I (intermediate) and R (rough) types. The existence of other forms of a relatively unstable character in the S to R conversion is recognized.

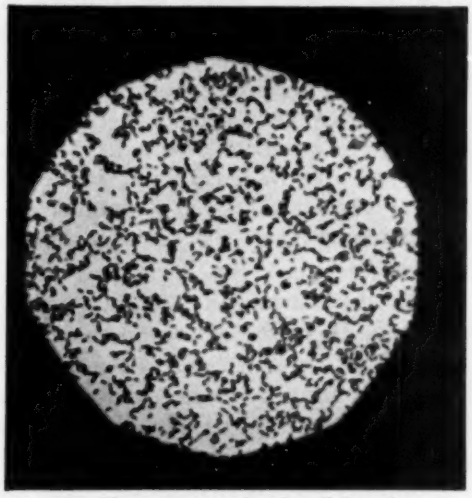


Fig. 6. Photomicrograph of cellular elements making up reverted W colonies (x 1,440).

3) Associated changes in antigenicity and virulence, as well as slight variations in biochemic activity, become increasingly pronounced as the I to R conversion progresses toward the R stage.

4) An unusual dissociative W form, differing in its colonial and cellular morphology from the usual Brucella variants, has been observed in aged vaccine prepared from strain 19.

5) Significant differences exist in the serologic relationship of the W forms as compared with the typical smooth types.

6) Reversion of the W forms to their S prototypes was accomplished by passage through guinea pigs.

7) The reverted types were culturally,

Studies on Certain Filtrable Viruses. III. Longevity of Chorioallantoic and Comb-Tissue Fowl-Pox Virus in Vitro*

C. A. BRANDLY,† D.V.M., M.S., ROBERT GRAHAM, B.S., D.V.M., and C. C. MORRILL,‡ D.V.M., M.S.

Urbana, Ill.

THE PRODUCTION of satisfactory active immunity to pox in domestic fowls requires the proper application of an antigenic pox virus to healthy birds of suitable age. Cutaneous vaccination with viable virus vaccine induces a definite local reaction or response in susceptible birds, active immunity developing coincident to the local infection. The absence of a local reaction following approved methods of applying vaccine may result from a natural or acquired immunity or the use of a nonviable or nonantigenic virus.

Since certain lots of fowl-pox-virus vaccine have become inactive on cold storage (12°-16° C.) over periods of ten months or longer, studies were undertaken to ascertain the influence of various temperatures, desiccation, and partial vacuum on the duration of viability of chorioallantoic and comb-lesion fowl-pox virus.

The advantages of preserving biological products, as well as bacterial cultures, by dehydrating *in vacuo* and at freezing temperatures have been set forth by Shackell[1] and Hammer.[2] More recently Flosdorf and Mudd,[3, 4] described improved methods of vacuum desiccation of normal and convalescent human sera, animal antisera, enzymes, bacteria and other materials. These investigators report that complement may be preserved without detectable deterioration for at least ten months, and antisera may probably be preserved unaltered for a period of several years.

In an attempt to appraise the influence of temperature, pressure, dehydration and bacterial contamination on the viability of tissue virus in storage, fresh pox-tissue lesions were harvested from the chorioallantoic membranes of chick embryos and from the combs of chickens. Separate lots of the two types of pox-tissue viruses were then subjected in parallel to dehydration at room temperature and atmospheric pressure, at room temperature *in vacuo*,* and at the sublimation temperature of dry ice *in vacuo*. Parallel samples of experimental viruses prepared by these methods were sealed *in vacuo* and under atmospheric pressure for storage at 0-4° C. The effect of these factors on viability was measured at intervals by inoculating healthy susceptible chickens with a 1 per cent virus tissue suspension. The results suggest that such factors as freezing, drying and exhaustion of air pressure may favorably influence the

*From the Laboratory of Animal Pathology and Hygiene, University of Illinois.

†Resigned.

‡Assigned by the Illinois State Department of Agriculture to the Laboratory of Animal Pathology and Hygiene to assist in diagnostic work.

[1]Shackell, L. F.: An improved method of desiccation, with some applications to biological problems. Amer. J. Physiol., xxiv (1909), pp. 325-340.

*The tubes were sealed in an absolute pressure of 300μ or less, according to the McLeod gauge.

[2]Hammer, B. W.: A note on the vacuum desiccaton of bacteria. J. Med. Res., xxiv (1911), pp. 527-530.

[3]Flosdorf, E. W., and Mudd, S.: Procedure and apparatus for preservation in "lyophile" form of serum and other biological substances. J. Immunol., xxix (1935), pp. 389-425.

[4]*Ibid.*: An improved procedure and apparatus for preservation of sera, microörganisms and other substances—the cryochem-process. J. Immunol., xxxiv (1938), pp. 469-490.

(*Continued from preceding page*)

morphologically, antigenically and physiologically comparable with the smooth forms.

8) The value of competent supervision of *Br. abortus* vaccine cultures is emphasized and a basis for such control is established.

TABLE I—Potency of Chorioallantoic and Comb-Tissue Fowl-Pox Viruses*

Pox Virus		Method of Preparation				Potency Tests at 3-Month Intervals						
				Sealed								
Lot No.	Tissue	Moist	Dried	A. P.	In Vacuo	3	6	9	12	15	18	24
1	C.A.	+	—	—	+	—	—	—	—			
	Comb	+	—	—	+	—	—	—	—	—		
2	C.A.	+	—	+	—	—						
	Comb	+	—	+	—	—	—	—	—			
3	C.A.	—	RT	—	+	—						
	Comb	—	RT	—	+	—	—	—	—	—		
4	C.A.	—	RT	+	—	—						
	Comb	—	RT	+	—	—						
5	C.A.	—	RTV	—	+	—	—	—	—	—	—	—
	Comb	—	RTV	—	+	—	—	—	—	—	—	—
6	C.A.	—	RTV	+	—	—	—	—	—	—	—	—
	Comb	—	RTV	+	—	—	—	—	—	—	—	—
7	C.A.	—	CO_2V	—	+	—	—	—	—	—	—	—
	Comb	—	CO_2V	—	+	—	—	—	—	—		
8	C.A.	—	CO_2V	+	—	—	—	—	—	—		
	Comb	—	CO_2V	+	—	—	—	—	—	—	—	—

*All tubes of virus were stored at a temperature of 0-4° C.
V = *in vacuo;* CO_2V = dried at sublimation temperature of dry ice *in vacuo;* C.A. = chorioallantois; R.T. = room temperature; A.P. = atmospheric pressure.

keeping qualities of the virus maintained at 0-4° C.

Preparation and Storage of Experimental Viruses

In the preparation of the viruses, duplicate series were made with freshly harvested chorioallantoic and comb-tissue viruses. The tissues were first ground in sterile mortars and a number of small pyrex tubes each containing 0.2 Gm. of fresh virus tissue were prepared for each of the eight lots of each type of tissue. The tubes in lot 1 were not desiccated, but were evacuated and sealed. Those in lot 2 were neither desiccated nor evacuated, but simply sealed. All the remaining lots were desiccated. The tubes in lot 3 were dried at room temperature, evacuated and sealed, while those in lot 4 were dried at room temperature and sealed without evacuation. The tubes in lots 5 and 6 were dried *in vacuo* at room temperature, those of lot 5 then being sealed *in vacuo* and those of lot 6 at atmospheric pressure. The tubes of lots 7 and 8 were dried *in vacuo* at the sublimation temperature of CO_2 (*i.e.*, in dry ice), those of lot 7 then being sealed *in vacuo* and those of lot 8 at atmospheric pressure. (See table I.) All tubes were sealed off in an oxygen flame. They were stored at a temperature of 0-4° C.

Results of Virus Viability Tests

At intervals of three months for 1½ years, and six months later terminating a twenty-four month observation period, a 1 per cent suspension of each lot of vaccine was applied to three susceptible chickens varying in age from 2 to 4 months. Fresh tubes were used for each test and were discarded after opening. The feathers were parted on the right and left shoulder and the chorioallantoic and comb-tissue vaccines, respectively, were introduced by a 4-ply needle which made four separate skin punctures, or, in some cases, four sticks were made with an 18-gauge hypodermic needle. Viability of vaccine was appraised on the basis of a typical local reaction at the points vaccine was applied. The inoculated birds were examined 7, 14 and 21 days or oftener following the application of the vaccine. After three weeks all vaccinated birds were artificially inoculated

with virulent fowl-pox virus to measure the protection induced by the experimental vaccine. Inoculated fowls that proved resistant to potent fowl vaccine were regarded as immune. The potency of virus employed in determining the presence or absence of immunity was established by simultaneously inoculating susceptible fowls.

On the basis of these observations the moist unaltered chorioallantoic tissue virus (lot 1) sealed and stored *in vacuo* remained viable for 12 months but was nonviable at 15 months, while moist comb virus sealed and stored *in vacuo* was viable at 15 months but nonviable at 18 months. Fresh moist chorioallantoic tissue and comb-tissue virus (lot 2) sealed in parallel at atmospheric pressure remained viable for 6 and 15 months, respectively. In lot 3, chorioallantoic tissue virus dried at room temperature and sealed *in vacuo* was viable at 6 months and nonviable at 9 months while comb-tissue virus dried at room temperature and sealed *in vacuo* was viable at 18 months and nonviable at 24 months. In lot 4, fowl-pox-tissue viruses (chorioallantoic and comb) dehydrated at room temperature and sealed at atmospheric pressure were viable at 6 months and nonviable at 9 months. In lots 5 and 6, tissue viruses dried at room temperature *in vacuo* and sealed *in vacuo* and at atmospheric pressure were viable after 24 months. In lot 7, the chorioallantoic and comb-tissue viruses dehyrated at the sublimation temperature of dry ice *in vacuo* and sealed *in vacuo* remained viable for 24 and 18 months, respectively. In lot 8, the chorioallantoic and comb-tissue virus dehydrated at sublimation temperature of CO_2 *in vacuo* and stored at atmospheric pressure remained viable for 18 and 24 months, respectively. (See table I.)

SUMMARY

Evidence is presented to show that drying of fowl-pox virus at room temperature *in vacuo* apparently lengthens the survival time of the virus *in vitro* when stored at a temperature of 0-4° C. as compared with that resulting from drying at room temperature without vacuum. No appreciable difference in antigenic properties was observed in viruses stored for 24 months *in vacuo* and at atmospheric pressure at 0-4° C. after drying *in vacuo* at room temperature. No advantage of dehydration at the sublimation temperature of dry ice over dehydration at room temperature appeared within a period of 24 months, as judged by viability.

In four instances the longevity of the comb-tissue virus was greater than that of the parallel chorioallantoic virus; in one instance the reverse was true. The egg-propagated virus would, nevertheless, seem to be a superior product to the comb-lesion virus in view of the advantages observed by Brandly and Dunlap[5] in previous studies, namely, greater concentration and freedom from bacterial contamination.

[5]Brandly, C. A., and Dunlap, G. L.: Studies on certain filtrable viruses. II. Immunization against fowl pox with fowl- and pigeon-pox viruses cultivated *in vivo* and *in vitro*. J.A.V.M.A., xcv (1939), pp. 340-349.

Preliminary Studies on the Blood Chemistry of the Fox*

E. H. SPITZER, B.S., A. I. COOMBES, M.A., and W. WISNICKY, D.V.M.

Madison, Wis.

RECENT SURVEYS of the fur industry in the United States showed that there were 272,527 silver fox pelts produced in this country during 1940. As a result of the wide variation in rations used by fur farmers, nutritional problems along with infectious diseases have played an important rôle in domestic fur production.

Since the fox is not commonly used as an experimental animal, only a few studies involving the hematopoietic system have been reported in the literature. Among these are included the work of Kennedy,[1] who made a study of the blood elements of normal foxes, and Smith,[2] who has reported some of the normal blood indices of foxes and minks. This investigation was undertaken to establish average blood indices for normal growing and mature foxes with reference to hemoglobin, hematocrit, erythrocyte counts, serum calcium and total inorganic phosphorus. These data should give indices for blood chemistry that can be used as a reference for interpreting the results of experiments involving animals used in nutritional research as well as in disease studies and for diagnostic purposes. Since the silver fox is a color phase of the red, both types were studied in this investigation to see whether or not data obtained from one phase would be applicable to the other.

METHODS

Blood was obtained from the mature silver foxes by cardiac puncture. While the animal was held in a vertical position and the heart palpated, a 13-gauge needle was inserted at a point about 1 to 2 cm. to the right of the sternum. Samples for serum calcium and inorganic phosphorus determinations were drawn into a clean, dry, 15-cc. centrifuge tube. Since the samples were taken during the pelting season, these animals were then sacrificed by bleeding to death, the remaining blood being drawn into the non-shrinking oxalate mixture described by Wintrobe.[3]

Blood from the growing red foxes also was obtained by cardiac puncture. An 18-gauge needle, fitted with a 30-cc. glass syringe, was inserted into the heart and 30 cc. of blood was drawn into the syringe, 15 cc. of which was expelled into a clean, dry, 15-cc. centrifuge tube for serum calcium and inorganic phosphorus determinations and the remainder into an oxalated flask as above. The samples were placed in a refrigerator room at 34-35° F. within one-half hour after sampling.

Hemoglobin, hematocrit and erythrocyte measurements were made within eight to ten hours after sampling. Hemoglobin determinations were made by a method adapted to the Evelyn photoelectric colorimeter[4] and the percentage volumes of packed red cells were determined by means of the Wintrobe hematocrit tube.[5] Hayem's solution was used to dilute the blood for the erythrocyte counts. A Levy counting chamber with improved Neubauer rulings was employed. Serum calcium was estimated by the method reported by Clark and Collip,[6] and inorganic phosphorus was determined by the colorimetric method of Fiske and Subbarow,[7] which was adapted to the Evelyn photoelectric colorimeter in this laboratory. Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration calculations were made by the method suggested by Wintrobe.[8]

---

*From the departments of biochemistry and veterinary science, College of Agriculture, University of Wisconsin.

[1]Kennedy, A. H.: A graphical study of the blood of normal foxes. Canadian J. Res., xii (1935), p. 796.

[2]Smith, S. E.: Blood glucose, plasma inorganic phosphorus, plasma calcium, hematocrit, and bone ash values of normal minks *(Mustela vison)* and foxes *(Vulpes fulva)*. Cornell Vet., xxxi (1941), p. 56.

[3]Wintrobe, M. M., Shumacker, H. B., and Schmidt, W. J.: Values for number, size and hemoglobin content of erythrocytes. Amer. J. Physiol., cxiv (1936), p. 502.

[4]Evelyn, K. A.: A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., cxv (1936), p. 63.

[5]Wintrobe, M. M.: Macroscopic examination of the blood. Amer. J. Med. Sci., clxxxv (1933), p. 58.

[6]Clark, E. P., and Collip, J. B.: A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., lxiii (1925), p. 461.

[7]Fiske, C. H., and Subbarow, Y.: The colorimetric determination of phosphorus. J. Biol. Chem., lxvi (1925), p. 375.

[8]Wintrobe, M. M.: The direct calculation of the volume and hemoglobin content of the erythrocyte. Amer. J. Clin. Path., i (1931), p. 147.

TABLE I—Blood Picture of the Normal Mature Silver Fox

Fox No.	Hb. (Gm. per 100 cc.)	Ht. (%)	RBC (Million per cmm.)	Corp. Vol. (Cubic Microns)	Corp. Hb. (Micro-micrograms)	Corp. Hb. Conc. (%)	Serum Ca (Mg. %)	Inorganic P (Mg. %)
1	15.2	58	8.5	68	18.0	26	12.5	6.1
2	13.9	54	7.6	71	18.3	26	13.2	5.5
3	15.3	63	7.6	84	20.0	24	13.2	6.4
4	14.6	53	7.7	69	17.7	26	12.7	6.6
5	15.8	62	8.4	74	18.8	25	12.5	6.6
6	14.5	59	8.1	73	17.9	25	13.4	6.8
7	14.8	61	7.4	82	20.0	24	14.0	6.7
8	14.4	58	7.9	74	18.2	25	12.8	5.1
9	14.9	63	8.1	78	18.4	24	10.4	5.6
10	16.1	63	8.1	78	20.0	26	11.9	5.0
11	14.5	55	8.1	79	18.0	26	13.1	4.8
12	16.1	64	8.1	61	20.0	25	12.7	5.9
Average	15.0	59	7.97	74.8	18.77	25.1	12.7	5.97

RESULTS

The data obtained from the mature silver foxes are presented in table I and those data from the maturing red foxes are shown in table II. By reference to table I, the average hemoglobin concentration of the mature silver fox is 15.0; the average percentage volume of packed red cells is 59 and the average erythrocyte count is 7.97. The average values for mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration are 74.8, 18.77, and 25.1, respectively. The average serum calcium and inorganic phosphorus indices are 12.7 and 5.97, respectively.

With reference to the maturing red fox, calculations from table II demonstrate that the concentration of hemoglobin, percentage volume of packed red cells and erythrocyte counts increase until approximately 10 months of age, at which time the blood indices approach those obtained from the mature silver fox (table I). The average concentration of hemoglobin increased from 12.6 at seven months of age to 16.5 at 10 months of age; the percentage volume of packed red cells increased from 47 to 52 and the erythrocyte counts increased from 7.5 to 8.3. Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration remained relatively constant; the same can be said for serum calcium, while a slight decrease was noted in total inorganic phosphorus between 8 and 10 months of age.

DISCUSSION

The average hemoglobin, hematocrit and erythrocyte indices of the mature silver fox

TABLE II—Normal Blood Picture of the Maturing Red Fox

Age in Months	Fox No.	Hb. (Gm. per 100 cc.)	Ht. (%)	RBC (Million per cmm.)	Corp. Vol. (Cubic Microns)	Corp. Hb. (Micro-microgr\ms)	Corp. Hb. Conc. (%)	Serum Ca (Mg. %)	Inorganic P (Mg. %)
7	A-8	12.8	48	7.9	61	16.5	27	10.8	6.1
	A-13	12.3	48	7.3	60	16.8	28	10.3	6.1
	A-16	12.6	45	7.2	63	17.5	28	11.2	5.7
	A-20	12.6	48	7.6	63	16.6	26	10.8	6.0
	A-38	12.6	48	7.6	64	17.2	27	11.9	7.1
Average		12.6	47	7.5	62	16.9	27	11.0	6.2
8	A-8	14.8	56	7.9	71	16.6	26	10.1	5.9
	A-13	13.5	48	7.4	65	18.2	29	10.1	5.9
	A-16	14.8	52	7.5	69	19.7	28	10.2	6.3
	A-20	15.6	55	8.6	64	18.1	28	10.2	6.2
	A-38	15.8	46	8.2	56	19.3	28	10.2	6.8
Average		14.9	51	7.9	65	18.4	28	10.2	6.2
10	A-8	17.5	53	8.5	52	20.6	33	11.2	5.6
	A-13	15.8	48	7.7	62	20.5	33	11.1	5.7
	A-16	16.9	53	8.5	62	20.0	32	11.6	5.8
	A-20	16.0	54	8.5	60	18.9	29	10.3	5.4
	A-38	16.1	51	8.4	61	19.2	26	11.1	5.9
Average		16.5	52	8.3	59	19.8	31	11.1	5.7

are distinctly higher than those in the adult dog. In a statistical analysis of 251 determinations on 34 dogs, Bruner and Wakerlin[9] reported that the average erythrocyte count is 6.45 million per cmm.; average hemoglobin and hematocrit determinations were 13.56 Gm. per 100 cc. of blood and 44.28 per cent, respectively. These data are significantly lower than our results (table I). The same authors reported average figures for mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration to be 68.9, 21.1 and 30.7, respectively, which show no significant variation from our results. It seems, therefore, that the values for these indices are essentially the same for both species.

The number of erythrocytes in young foxes is below the average number for the mature fox. These results are in agreement with the literature with reference to the dog.[10, 11] As the number of red blood cells increases with age, the concentration of hemoglobin and percentage volume of packed red cells also increase.

These results indicate that there is no significant difference between the normal blood indices of the mature red fox and those of the mature silver fox. It seems, therefore, that data obtained from the red fox may be used in the interpretation of the blood chemistry of the silver fox.

There appears to be a slight fall in total inorganic phosphorus with age. A similar condition has been noted in dogs,[12] sheep[13] and cattle.[14]

Further studies on the blood chemistry of the fox are in progress in this laboratory. Some of the indices being studied include blood sugar, uric acid, plasma iron, copper, vitamin A, phosphatase, urea, creatinine, non-protein nitrogen and total protein. Results of these studies are as yet incomplete.

SUMMARY

1) A study of the blood chemistry of red and silver foxes was undertaken to establish certain of the normal blood indices which might be used as a reference in nutritional and disease studies and for diagnostic purposes.

2) The data obtained demonstrate that the average erythrocyte count for the mature silver fox is 7.97 million per cmm.; the percentage volume of packed red cells is 59 and the hemoglobin concentration is 15 Gm. per 100 cc. of blood.

3) Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration calculations are 74.8 cu. μ, 18.77 micromicrograms, and 25.1 per cent, respectively.

4) There is a proportional increase with age of the hemoglobin concentration, percentage volume of packed red cells, and erythrocyte counts in the maturing red fox.

5) Age apparently has no significant effect on mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration.

6) There appears to be a slight decrease in total inorganic phosphorus as the fox reaches maturity.

7) The data obtained from the red fox can be used in interpreting the blood chemistry of the silver fox.

ACKNOWLEDGMENTS

The authors wish to express sincere appreciation to Mr. W. E. Beck, Rolling Prairie, Wis.; to the Capitol Fox and Fur Ranch, Madison, Wis.; and to the Nieman-Stephenson Co. of Cedarburg, Wis., for providing certain of the animals from which blood specimens were obtained.

[9] Bruner, H. D., and Wakerlin, G. E.: The blood picture of the normal dog. Proc. Soc. Exp. Biol. & Med., xxxvi (1937), p. 667.

[10] Morris, M. L., Allison, J. B., and Green, D. F.: Blood cytology of the normal dog. J. Lab. & Clin. Med., xxv (1940), p. 353.

[11] Scarborough, R. A.: The blood picture of normal laboratory animals. Yale J. Biol. & Med., iii (1930), p. 359.

[12] Anderson, H. D., and Elvehjem, C. A.: Variations in the blood calcium and phosphorus with the age of the dog. J. Biol. Chem., cxxxiv (1940), p. 217.

[13] Malan, A. I.: Studies in mineral metabolism. VIII. Comparison of phosphorus partition in the blood of the calf foetus, sheep foetus, and lambs, with corresponding maternal blood. J. Agr. Sci., xviii (1928), p. 397.

[14] Green, H. H., and Macaskill, E. H.: Studies in mineral metabolism. VI. Comparison of the blood of the cow and calf in respect to mineral constituents. J. Agr. Sci., xviii (1938), p. 384.

Data and Bibliography on Some Nitrogenous Constituents of Normal Dog's Blood*

JAMES B. ALLISON, Ph.D., HERMAN O. DRESKIN, M.S., and M. L. MORRIS, D.V.M.

New Brunswick, N. J.

VARIATIONS from the normal in the concentrations of some of the nitrogenous constituents in the blood are valuable aids in the quantitative determinations of many abnormalities in the dog. Before these abnormalities can be quantitatively evaluated, however, it is necessary to know the normal variations of the protein and non-protein forms of nitrogen in the blood.

Data have been accumulated on the total protein, "albumin," "globulin" and non-protein nitrogen in the blood sera and on the urea nitrogen, creatinine, and uric acid in the blood of normal dogs. The animals were well bred, from good homes and free from abnormalities. Their state of health was determined by examinations made under the routine conditions of an animal hospital. The data obtained on these dogs can be compared with the numerous data in the literature coming from experimental laboratories where the animals were generally kept under different environmental conditions. In this way "normals" can be established to be used as references for the evaluation of data obtained from the analysis of blood of abnormal animals in animal hospitals.

METHODS

Blood samples were taken with a minimum of stasis from the jugular or radial veins. Common clinical laboratory procedures were used for analysis. The Koch and McMeekin[1] micro-Kjeldahl method was used for the determination of nitrogen. In most cases the determinations for proteins were repeated on the sera, the Pregl micro-Kjeldahl method being used. The albumin and globulins were separated by the method of Howe,[2] by the procedure described by Bodansky and Fay.[3] For the determination of non-protein nitrogen in the sera the proteins were precipitated by the method of Folin and Wu.[4] The urea nitrogen, creatinine and uric acid were determined on unlaked whole blood filtrate recommended by Folin and Svedberg.[5] Urea nitrogen was determined on the filtrate by the methods of Karr[6] and of Van Slyke and Cullen.[7] Preformed creatinine was determined by the method of Folin and Wu[4] and uric acid by Folin's procedure.[8, 9]

RESULTS AND COMMENTS

The data obtained from the analyses of the sera of 20 normal dogs for total protein, "albumin," "globulin," non-protein nitrogen, and from the analyses of the whole blood of other groups of 20 normal dogs for urea nitrogen, creatinine and uric acid are recorded in table I.

The average values recorded in this table are in close agreement with averages obtained from numerous data in the literature on the concentration of proteins in the blood sera of dogs. For example, data from the literature,[10-32] representing analyses on the sera of over 200 dogs, were averaged. These average values are 6.2 Gm. of protein, 3.8 Gm. of "albumin" and 2.3 Gm. of "globulin" per 100 cc. of serum. The average albumin/globulin ratio for these data is 1.65. The maximum and minimum values found in these data, however, are probably outside the normal range for dogs received at an animal hospital. Concentrations of protein as high as 8 Gm. and as low as 3 Gm. per 100 cc. of serum are reported. The albumin/globulin ratios vary between 4.4 and 0.46. The latter high variation is due in part to the use of different technics to separate the "globulins" from the "albumins."

The majority of the values recorded in

*From the Department of Physiology and Biochemistry, Rutgers University, and the Raritan Hospital for Animals; a contribution from the Bureau of Biological Research, Rutgers University.

Assistance in the preparation of these materials was furnished by the personnel of Work Projects Administration official project No. 65-1-22-477.

The data on serum proteins were presented by Mr. Dreskin in a thesis as partial fulfilment for the degree of Master of Science at Rutgers University.

TABLE I — Proteins and Non-Protein Nitrogen in Blood Sera of 20 Normal Dogs. Urea Nitrogen, Creatinine and Uric Acid in Bloods of Other Groups of 20 Normal Dogs

SERUM	MAXIMUM GM. PER 100 CC.	MINIMUM GM. PER 100 CC.	AVERAGE GM. PER 100 CC.	MEAN DEVIATION
Total protein	7.10	5.64	6.20	0.37
Albumin	4.52	3.28	4.00	0.20
Globulin	2.97	1.45	2.20	0.30
N. P. N.	0.058	0.024	0.035	0.006
Albumin Globulin	2.98	1.35	1.82	0.32
BLOOD	MG. PER 100 CC.	MG. PER 100 CC.	MG. PER 100 CC.	
Urea nitrogen	22.6	10.0	15.5	2.4
Creatinine	1.7	1.0	1.36	0.13
Uric acid	0.36	0.0	0.21	0.09

the literature for non-protein nitrogen in the blood sera compare favorably with the data given in table I. Wells[23] reported an average of 35.2 mg. of non-protein nitrogen per 100 cc. of serum. The concentration of non-protein nitrogen in the blood is, however, effected by the diet. Values lower than those shown in table I are encountered when the dog receives a diet restricted in protein. Non-protein nitrogen and urea nitrogen will increase above the so-called normal values if a high protein diet is fed. An increase in these forms of nitrogen also has been reported[33] in animals which are fasting.

Most of the determinations reported in the literature for urea nitrogen and creatinine have been made upon laked blood, but hemolysis does not change the concentration of urea nitrogen or of creatinine significantly. Leichsenring, Biester, Hornig, Furnar, Foss and Routt[34] reported averages, based on the analysis of the blood of 28 dogs, of 11.04 mg. of urea nitrogen and 1.52 mg. of creatinine. Morgulus and Edwards[33] reported averages based on six dogs of 12.9 mg. of urea nitrogen and 1.6 mg. of creatinine. Haden and Orr[35] analyzed the bloods of 200 dogs and found an average of 11.7 mg. of urea nitrogen and 1.5 mg. of creatinine. Urea nitrogen varied in these data from 5.1 to 42.1 mg. and creatinine from 0.5 to 2.6 mg. Such extremes are, however, seldom encountered at the hospital in normal dogs. More than 1,000 urea-nitrogen determinations have been made in the hospital, for example, and in the majority of cases a blood-urea nitrogen over 23 mg. per 100 cc. of blood is due to nitrogen retention from kidney damage or from some other abnormality.

Benedict[36] demonstrated that the end product of urine metabolism in the Dalmatian Coach Hound is uric acid, but Mann and Magath,[37] Bollman, Mann and Magath[38] and others have shown that in other breeds almost all of the uric acid is converted into allantoin by the liver. The highest concentration of uric acid which we have found in any dog other than the Coach Hound is 0.7 mg. per cent.[39] Higher concentrations of uric acid in the blood of normal dogs sometimes recorded in the literature are probably due to the nonspecificity of the method of analysis used. Blauch and Koch,[40, 41] for example, by using uricase to destroy the acid, found that the concentrations of uric acid varied from 0 to 0.5 mg. per cent in ten samples of normal dogs' blood.

Bibliography

[1]Koch, T. G., and McMeekin, T. L.: A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Amer. Chem. Soc., xlvi (1924), p. 2066.

[2]Howe, P. E.: The determination of proteins in blood—a micro method. J. Biol. Chem., xlix (1921), p. 109.

[3]Bodansky, M., and Fay, M.: Laboratory Manual of Physiological Chemistry. (4th ed., John Wiley and Sons, Inc., New York, 1938.)

[4]Folin, O., and Wu, H.: A system of blood analysis. J. Biol. Chem., xxxviii (1919), p. 81.

[5]Folin, O., and Svedberg, A.: Diffusible nonprotein constituents of blood and their distribution between plasma and corpuscles. J. Biol. Chem., lxxxviii (1930), p. 715.

[6]Karr, W. G.: A method for the determination of blood urea nitrogen. J. Lab. & Clin. Med., ix (1924), p. 329.

[7]Van Slyke, D. D., and Cullen, G. E.: A permanent preparation of urease and its use for rapid and accurate determination of urea. J.A.M.A., lxii (1914), p. 1558.

[8]Folin, O.: Unlaked blood as a basis for blood analysis. J. Biol. Chem., lxxxvi (1930), p. 179.

[9]*Ibid.*: The preparation of sodium tungstate free from molybdate, together with a simplified process of the preparation of a correct uric acid reagent (and some comments). J. Biol. Chem., cvi (1934), p. 311.

[10]Burckhardt, A. E.: Beiträge zur Chemie und Physiologie des Blutserums. Arch. f. Exp. Path. u. Phar., xvi (1883), p. 322.

[11]Lewinski, J.: Beobachtungen über den Gehalt des Blutplasmas an Serumalbumin, Serumglobulin und Fibrinogen. Arch. f. Physiol. Pflüger, c (1903), p. 100.

[12]Moll, L.: Ueber Blutveränderungen nach Eiwei-Binjektionen. Beitr. Chem. Phys. u. Path., iv (1904), p. 578.

[13]Morawitz, P.: Beobachtungen über den Wiedererersatz der BlutewelBkörper. Beitr. Chem. Phys. u. Path., vii (1906), p. 153.

[14]Cervello, C.: Quoziente Proteico del siero di

Sangue e suo significato Fisiologico. Archivo Farmacol. Eterpeut., xiv (1908), p. 391.

[15]Woolsey, J. H.: Studies in the blood relationship of animals as displayed in the composition of the serum proteins. II. A comparison of the sera of the ox, sheep, hog, goat, dog, cat and guinea pig with respect to their content of various proteins. J. Biol. Chem., xiv (1913), p. 433.

[16]Jewett, R. M.: Studies in the blood relationships of animals as displayed in the compositions of the serum proteins. V. The percentage of non-proteins in the sera of certain animals and birds. J. Biol. Chem., xxv (1916), p. 21.

[17]Hurwitz, S. H., and Whipple, G. H.: Studies on blood proteins. II. The albumin-globulin ratio in experimental intoxications and infections. J. Exp. Med., xxv (1917), p. 231.

[18]Bodansky, M., Morse, S. W., Kiech, V. C., and Bramkamp, R. B.: The distribution of proteins in the blood in experimental anemia. J. Biol. Chem., lxxiv (1927), p. 463.

[19]Matthew, C. W.: The plasma proteins of normal dogs. J. Biol. Chem., lxxiv (1927), p. 557.

[20]Barker, H. M., and Kirk, E. J.: Experimental edema (nephrosis) in dogs in relation to edema of renal origin in patients. Arch. Int. Med., xlv (1930), p. 319.

[21]Bonsmann, M. R.: Verhalten des kolloidosmotischen Drucken und der Eiweissfraktionen im Serum auf der Höhe der Tonexhinwirkung beim Hunde. Arch. f. Exp. Path. u. Phar., clxiv (1932), p. 211.

[22]Cuvelier, R., and Patoir, G.: La régénération des albumines du sérum sanguin après las saignée chez le chien. Compt. Rend. Soc. Biol., cxi (1932), p. 16.

[23]Wells, H. S.: The concentration and osmotic pressure of the proteins in blood serum and in lymph from the lacteals of dogs. Amer. J. Physiol., ci (1932), p. 421.

[24]Weech, A. A., Goettsch, E., and Reeves, E. B.: The proteins of blood and subcutaneous lymph in dogs. J. Clin. Investig., xii (1933), p. 1021.

[25]Field, M. E., Leigh, O. C., Heim, J., and Drinker, C. K.: The protein content and osmotic pressure of blood serum and lymph from various sources in the dog. Amer. J. Physiol., cx (1934), p. 174.

[26]Moen, J. K., Medes, C., and Chalek, I.: Relative effects of diathermy and infection on the plasma proteins, plasma viscosity and suspension stability of the blood in dogs. J. Lab. & Clin. Med., xix (1934), p. 571.

[27]Weech, A. A., Goettsch, E., and Reeves, E. B.: Nutritional edema in the dog. Development of hypoproteinemia on a diet deficient in protein. J. Exp. Med., lxi (1935), p. 299.

[28]Heim, J. W.: On the chemical composition of lymph from subcutaneous vessels. Amer. J. Physiol., ciii (1933), p. 553.

[29]Fiessinger, N. A., Gajdos, A., and Panayotopaulos, E.: L'équilibre protidique du sérum au cours du choc histaminique. Compt. Rend. Soc. Biol., cxxiii (1936), p. 967.

[30]Wladasch, A.: Das Verhältnis von Albumin zu Globulin im Blutserum Gesumder Haustiere. Biochem. Zeit., cclxxxvii (1936), p. 337.

[31]Hastings, A. B., and Eichelberger, L.: The exchange of salt and water between the muscle and blood. I. The effect of an increase in total body water produced by the intravenous injection of isotonic salt solutions. J. Biol. Chem., cxvii (1937), p. 73.

[32]Robinson, H. W., Price, J. W., and Hogden, C. G.: The estimation of albumin and globulin in bloom serum. I. A study of the errors involved in the filtration procedure. J. Biol. Chem., cxx (1937), p. 481.

[33]Morgulus, S., and Edwards, A. C.: Chemical changes in the blood during fasting and subsequent refeeding. Experiments on dogs. Amer. J. Physiol., lxviii (1924), p. 477.

[34]Leichsenring, J. M., Biester, A., Honig, H. H., Furnas, S. M., Foss, E. S., and Routt, M. V.: Blood regeneration studies. II. Observations on the blood of normal dogs with special reference to the measurement of volume, erythrocytes, leucocytes and nitrogenous constituents. Amer. J. Physiol., xcix (1931-32), p. 391.

[35]Haden, R. L., and Orr, T. J.: Chemical findings in the blood of the normal dog. J. Biol. Chem., lxv (1925), p. 479.

[36]Benedict, S. R.: Uric acid in its relation to metabolism. J. Lab. & Clin. Med., ii (1916), p. 1.

[37]Mann, F. C., and Magath, T. B.: Studies in the physiology of the liver. III. The nitrogen constituents of the blood following removal of the liver. Amer. J. Physiol., lv (1921), p. 36.

[38]Bollman, J. L., Mann, F. C., and Magath, T. B.: Studies on the physiology of the liver. X. Uric acid following total removal of liver. Amer. J. Physiol., lxxii (1925), p. 629.

[39]Allison, J. B., Green, D. F., and Morris, M. L.: Clinical laboratory methods in small animal practice. J.A.V.M.A., xciii (1938), p. 176.

[40]Blauch, M. B., and Koch, F. C.: Studies on enzymatic formation and destruction of uric acid in mammalian blood. Proc. Soc. Exp. Biol. & Med., xxxviii (1938), p. 638.

[41]*Ibid.*: Application of the uricase method to the study of changes *in vitro* in the uric acid content of certain mammalian bloods. J. Biol. Chem., cxxx (1939), p. 455.

A New Record of a Deer Parasite for California*

R. F. ANNEREAUX, B.S.

Sacramento, Calif.

NUMEROUS PARASITES of domestic animals have been encountered in wild deer. Reports of parasitisms in this species are, therefore, of extreme interest to parasitologists and veterinarians because of the economic importance of the deer as a reservoir of animal parasites. More attention should be paid to deer along with other wild animals as helminth hosts. It is apparent, from a search of the literature, that little information is available on deer parasites in California. Recent studies by workers in the Zoölogical Division, Bureau of Animal Industry, U. S. Department of Agriculture, have greatly increased our knowledge of this important group of parasites. Such species as *Chabertia ovina, Cooperia curticei, Dictyocaulus filaria, Haemonchus contortus, Nematodirus filicollis, Ostertagia circumcincta, Trichostrongylus axei, Fasciola hepatica, Monezia expansa*, and others, normally parasitic in cattle or sheep, have been reported as occurring in deer.

On September 24, 1940, a local sportsman, Mr. Leal Garner, brought to the Animal Pathology Laboratory a section of skin removed from the region of the hock joint of a deer. Numerous nematodes lying in loops were seen in channels of the subcutaneous connective tissue. Mr. Garner stated that these parasites also were noted under the skin in the brisket region. The deer, a mule deer (*Odocoileus hemionus*) was killed approximately eight miles east of Truckee, Nevada county, Calif.

Extraction of the nematodes from the subcutaneous fascia channels proved to be quite difficult; of the total number extracted, only four female and two male worms were secured entire. The worms are identified as specimens of *Onchocerca cervipedis*.

O. cervipedis was described by Wehr and Dikmans[1] in 1935, from material obtained from deer (*Odocoileus virginianus, O. columbianus* and probably *O. hemionus*) in Montana and British Columbia. "The parasites," to quote the above investigators, "are reported as having been collected from the subcutaneous tissue of the hindquarters, foot and ankle of deer. According to Mr. Rush, the adult worms remain extended in the subcutaneous connective tissue of the foot during the period of their development. At maturity they coil up loosely and at the time the larvae are being discharged a puncture is formed in the skin and this gradually becomes a bad sore. Heavy infestations cause a swelling of the foot and a loosening and, finally, the detachment of the hoof."

The nematodes collected from the California deer agree in all essential characters with those described for *O. cervipedis*. However, it was possible to orient one specimen, a male, in such a way that a ventral view of the tail was available for observation. On the basis of these observations the author ventures to supplement the description of Wehr and Dikmans.

O. CERVIPEDIS WEHR AND DIKMANS

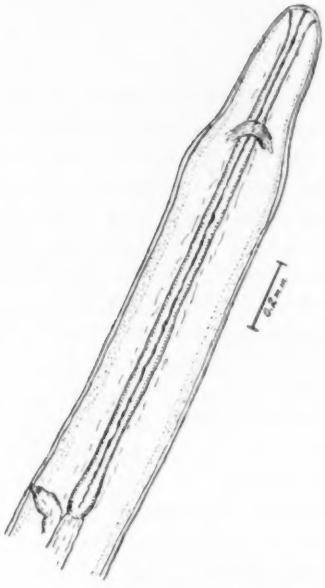
Description.—With the characters of the genus Onchocerca as revised by Wehr and Dikmans (1935). Long filiform worms. Cuticle with fine transverse striations, without cuticular annular thickenings. Slightly tapering at both ends. Anterior extremity with a pronounced swelling starting in the region of the nerve ring. Oral opening simple; esophagus long, narrow, without any noticeable increase in diameter in its posterior end; intestine only slightly larger than the esophagus.

Male: 40 mm. long; greatest width 224 μ across swelling in anterior extremity; diameter near mid-portion of body 211 μ. Nerve ring 268 μ from anterior extremity. Esophagus 1.07 mm. long. Spicules unequal and dissimilar. The long spicule 226 to 245 μ in length; heavy

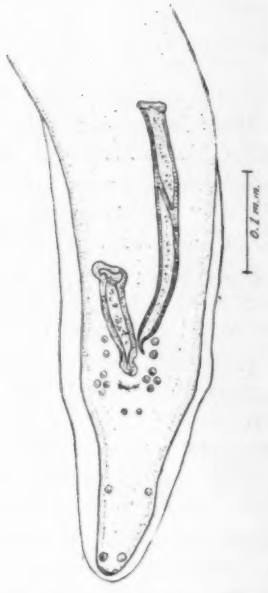
*From the Animal Pathology Laboratory, California State Department of Agriculture.

[1]Wehr, E. E., and Dikmans G.: New nematodes (Filariidae) from North American ruminants, Zoöl. Anz., cx (1935), pp. 202-208.

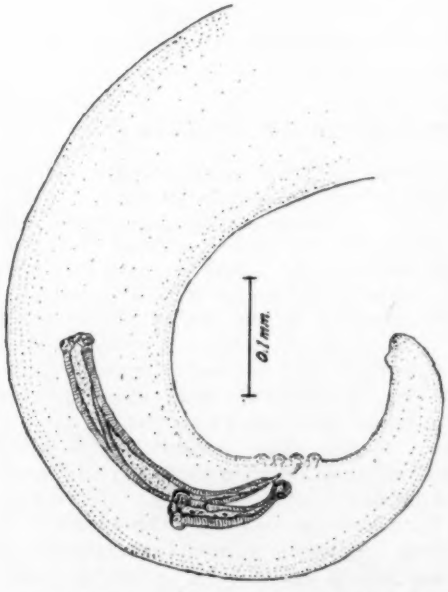
Onchocerca cervipedis Wehr & Dikmans



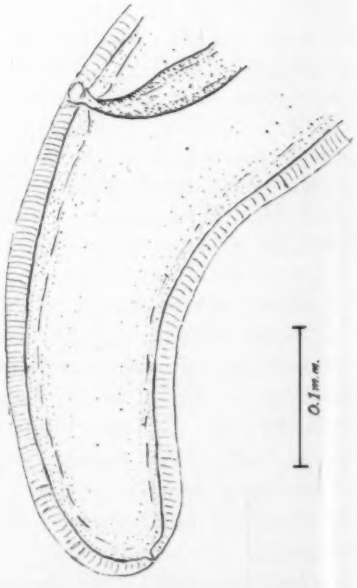
Anterior extremity of female.



Posterior extremity of male, lateral view.



Posterior extremity of male, ventral view.



Posterior extremity of female.

chitinized walls; a heavy chitinized ridge running diagonally across the anterior half of its length; the distal end tapering abruptly to a sharp point. The short spicule 112 to 120 μ in length; thick chitinized walls; in lateral view tapering toward the distal end, constricting and then expanding to form a rounded end. In lateral view, the posterior extremity appears to have four pairs of papillae in the area of the anal opening and one pair of large caudal papillae very close to the end of the tail. In ventral view a very different papillar pattern is observed. The papillae are arranged in the following manner: two pairs of adanal papillae, a group of four pairs of papillae in the region of the anus, one small pair of papillae near the mid-line anterior to but near the anus, one pair of papillae in the posterior part of the anterior half of the tail, and one large pair of papillae near the tip of the tail. In addition, there appears to be a row of small papillae transversing the end of the tail. Their exact number and conformation could not be determined. Length of tail 155 to 180 μ.

Female: Length 140 to 220 mm.; greatest width near center of body 345 μ. Nerve ring approximately 335 μ from anterior end. Esophagus 1.52 to 1.56 mm. long. Vagina near the union of esophagus and intestine. (In one specimen the vagina was at the union of the esophagus and intestine; in another specimen the vagina was located 431 μ anterior to esophagus-intestine junction.) Vagina divides into two uteri.

Except for minor differences in body measurements, the above description agrees in the major part with that of Wehr and Dikmans. This is particularly true of the spicule measurements, which are a safe criterion for differentiation between species. Wehr and Dikmans describe the short spicule and illustrate it as terminating in a hook. If the chitinization of the ventral end of the spicule is not pronounced, a hook-like appearance may be assumed. The above authors describe the caudal papillae as being in two groups: a first group of four pairs of adanal papillae and a second group of two pairs of papillae near the end of the tail. In lateral view of the specimens from California material, the first group is found, but only one pair of papillae is seen near the end of the tail. In ventral view, however, the second pair of papillae was found, but this pair was more cephalic in position.

COMMENT

Since the 1924 outbreak of foot-and-mouth disease in California, when thousands of deer were infected, the veterinary profession in this state has been constantly on the alert for a possible recurrence of this disease. A careful check has been made on all reports of sore mouths or hoofs in livestock. It is, therefore, of interest to note that *Onchocerca cervipedis* may be the cause of lameness in deer.

Swine Erysipelas in Turkeys*

A. S. ROSENWALD, B.S., D.V.M., and E. M. DICKINSON, D.V.M.

Corvallis, Ore.

SWINE ERYSIPELAS in turkeys was first reported by Jarosch.[1] A single case in a turkey was reported by Eber.[2] Beaudette and Hudson,[3] reporting an extensive outbreak observed in 1934, cited European reports of sporadic cases of the infection in various avian species. Graham, Levine and Hester[4] reported an extensive outbreak among a large duck population, while Waller[5] reported a case in quail.

In fairly rapid succession outbreaks in turkeys have been reported by Madsen,[6] Van Roekel, Bullis and Clarke,[7] Hoffman and Hinshaw,[8] Rosenwald and Dickinson[9] and Schlotthauer and Thompson[10] from widely separated areas in the United States.

This increasing recognition of swine erysipelas infection in birds and its economic importance to turkey growers emphasized the need for more information concerning the source of infection and possible preventive or control measures. This paper reports observations on 16 flocks of turkeys in which erysipelas occurred and results of field trials and laboratory studies on control measures and transmission.

For the sake of simplicity the term erysipelas as used in this paper refers to infection with *Erysipelothrix rhusiopathiae* unless otherwise stated.

ECONOMIC LOSS

As the mortality from erysipelas occurred just about market time in the fall or winter, the death of even one bird represented considerable financial loss.

The death loss was not the only economic waste due to erysipelas in turkeys. After sick individuals were removed, the apparently well birds from several of the flocks were marketed before the birds had reached optimum degree of finish and hence were sold at a less favorable price. Furthermore, some of the carcasses were either rejected or degraded because they did not dress well.

PUBLIC HEALTH ASPECTS OF TURKEY ERYSIPELAS

Obviously, there is a public health aspect in the sale of apparently well birds from a turkey flock in which erysipelas is occurring. Van Roekel *et al*[7] and Waller[5] have mentioned the possible importance of human erysipeloid contracted from birds. Klauder,[11-13] Klauder and Harkins[14] and

*Published as technical paper No. 356 of the Oregon State College Agricultural Experiment Station, contribution of the Department of Veterinary Medicine; presented before the Section on Poultry at the 77th annual meeting of the American Veterinary Medical Association, Washington, D. C., August 26-30, 1940.

[1]Jarosch, L. W.: Regarding a septicemia of turkeys. (Title trans.) Oesterreich. Monatsch. f. tier., xxxiii (1905), pp. 197-199. Cited by Beaudette and Hudson in J.A.V.M.A., lxxxviii (April 1936), pp. 475-488.

[2]Eber, A.: Septicemic erysipelas of birds. (Title trans.) Deut. Tierarztl. Wochnschr., xxix (1921), pp. 295-298. Cited by Beaudette and Hudson in J.A.V.M.A., lxxxviii (April 1936), pp. 475-488.

[3]Beaudette, F. R., and Hudson, C. B.: An outbreak of acute swine erysipelas infection in turkeys. J.A.V.M.A., lxxxviii (April 1936), pp. 475-488.

[4]Graham, R., Levine, N. D., and Hester, H. R.: *Erysipelothrix rhusiopathiae* associated with a fatal disease in ducks. J.A.V.M.A., xcv (Aug. 1939), pp. 211-216.

[5]Waller, E. F.: Erysipelothrix infection in quail. J.A.V.M.A., xcv (1939), pp. 512-513.

[6]Madsen, D. E.: An erysipelas outbreak in turkeys. J.A.V.M.A., xci (1937), pp. 206-208.

[7]Van Roekel, H., Bullis, K. L., and Clarke, M. K.: Erysipelas outbreaks in turkey flocks. J.A.V.M.A., xcii (1938), pp. 403-418.

[8]Hoffman, H. A., and Hinshaw, W. R.: Erysipelas in turkeys. Poultry Sci., xviii (1938), p. 443.

[9]Rosenwald, A. S., and Dickinson, E. M.: A report of swine erysipelas in turkeys. Cornell Vet., xxix (1939), pp. 61-67.

[10]Schlotthauer, C. F., and Thompson, L.: The occurrence of erysipelas in turkeys. J.A.V.M.A., xcvi (1940), pp. 103-104.

[11]Klauder, J. V.: Erysipeloid and swine erysipelas in man. J.A.M.A., lxxxvi (1926), pp. 536-541.

[12]*Ibid.:* Erysipeloid: bacillus of swine erysipelas infection—a disease of industry. J. Ind. Hyg., xiv (1932), pp. 222-234.

[13]*Ibid.:* Erysipeloid as an occupational disease. J.A.M.A., cxi (1938), pp. 1345-1348.

[14]Klauder, J. V., and Harkins, M. J.: Erysipeloid in the United States. J.A.M.A., xcvi (1931), pp. 1205-1209.

Klauder, Righter and Harkins[15] noted that human erysipeloid is an occupational disease. Russell and Lamb,[16] in reporting a fatal case, predicted that more cases in man will be recognized in the future, as the prevalence of erysipeloid is being publicized.

Lesions suggestive of erysipeloid have been reported by three persons closely associated with some of these outbreaks. However, no verified diagnosis of this infection in man due to contact with turkeys has been reported.

SWINE ERYSIPELAS IN OREGON

E. rhusiopathiae was first isolated by Muth[17] (1937) from a case of ovine polyarthritis. Rosenwald and Dickinson[9] isolated the infection from turkeys that same fall. Lytle and Derflinger[18] diagnosed erysipelas in a herd of swine by characteristic lesions and positive agglutination tests. Late in the summer of 1939, the organism was isolated from a vegetative endocarditis in a sow.

Within the past three years *E. rhusiopathiae* has been isolated once from sheep, once from hogs, and on 17 occasions (usually from more than one bird per occasion) from turkeys. Hence, in this state erysipelas infection must be regarded as a menace to turkeys approaching marketable age and condition.

In none of the recorded cases have turkeys and swine been in contact. In nearly half the outbreaks the birds ranged on "sheeped-over" ground. However, erysipelothritic arthritis has not been found in the sheep of the Willamette Valley, where these turkey losses occurred.

Since the first outbreak of erysipelas in the state, the recorded incidence of the disease in turkeys has increased. In 1938, five outbreaks were diagnosed, while eight outbreaks were encountered in 1939. For the sake of brevity, one case in a week-old poult reported by Rosenwald[19] is not included in this presentation.

THE ORGANISM

Erysipelas infection in turkeys was caused by a slender, highly pleomorphic, nonmotile rod, *E. rhusiopathiae*. The organism stained as clumps of curved, beaded or filamented Gram-positive organisms which decolorized easily. It grew readily, though sparsely, on ordinary culture media. Colony forms ranged from dewy, discreet, pin-point to opaque, flat, dry, pin-head type; all adhered tenaciously to solid media. Serum enhanced growth. The erysipelas organism grew especially well in deep stabs of semisolid medium, prepared by adding 0.5 per cent agar to difco tryptose-phosphate broth.

The strains of *E. rhusiopathiae* isolated from 16 outbreaks in turkeys fermented galactose, dextrose, lactose and levulose nonaerogenically. Lead-acetate agar was sometimes browned and l-xylose was occasionally fermented. Occasionally litmus milk was slightly acidified without coagulation. Typical test-tube-brush growth occurred in gelatin stabs incubated at room temperature.

Parenteral injection of the organism regularly killed mice *(Mus musculus)*, pigeons and turkeys. Guinea pigs and chickens survived such injections.

Murnane[20] feels that the erysipelas bacillus that causes ovine arthritis may be a different species from that causing the swine disease. Marsh,[21] Caudron,[22] Hart[23] and most workers feel that the organisms are identical. Our limited studies of or-

[15]Klauder, J. V., Righter, L. L., and Harkins, M. J.: A distinctive and severe form of erysipeloid among fish handlers. Arch. Dermat. & Syph., xiv (1926), pp. 662-678.

[16]Russell, W. O., and Lamb, M. E.: Erysipelothrix endocarditis; a complication of erysipeloid. J.A.M.A., cxiv (1940), pp. 1045-1050.

[17]Muth, O. H.: Personal communication (1937).

[18]Lytle, W. H., and Derflinger, E. R.: New hog disease, swine erysipelas, diagnosed. Dept. Agr. of Oregon Bul. 74 (1938), pp. 13-14.

[19]Rosenwald, A. S.: Swine erysipelas in a week-old turkey poult. J.A.V.M.A., xcvi (1940), pp. 268-269.

[20]Murnane, D.: Arthritis in lambs. Australian Vet. J., xiv (1938), pp. 23-35. Abst., Vet. Bul., ix, p. 369.

[21]Marsh, H.: The serological identity of strains of *Erysipelothrix rhusiopathiae* of ovine and porcine origin. J.A.V.M.A., lxxxii (1933), pp. 584-586.

[22]Caudron, M. F.: *Erysipelothrix rhusiopathiae* infection in sheep. (Title trans.) Thesis, Alfort (1936), p. 108. Abst., Vet. Bul., ix, p. 292.

[23]Hart, L.: The occurrence of swine erysipelas in New South Wales. Australian Vet. J., xiv (1938), pp. 12-15. Abst., Vet. Bul., xiv, p. 369.

ganisms isolated from sheep, swine and turkeys indicated that they are identical. These strains exhibited the same general characteristics as the *E. rhusiopathiae* strains isolated and described by other workers.

SYMPTOMS

Turkeys with erysipelas did not exhibit symptoms of a characteristic nature. The outbreaks started suddenly. Poisoning and

Fig. 1. Intramuscular hemorrhage in the pectoral muscles.

deaths due to stampede injuries or to mysterious predators were frequently suspected by owners. A few droopy birds, especially toms, were noted. Usually these somnolent individuals were easily aroused. However, some birds became very droopy with unsteady gait just before death.

MORBIDITY AND MORTALITY

The morbidity in flocks under observation was not very high. However, it was consistently noted that most of the sick birds died.

Mortality varied from the death of an occasional bird in good condition to a sudden loss of several birds within 24 hours. In the 16 outbreaks under observation mortality ranged from less than 1 per cent to nearly 12 per cent. Early sale of birds from affected flocks undoubtedly curtailed the loss in some of these flocks.

LESIONS

At autopsy most field cases were characterized by congestion and intramuscular and subpleural hemorrhages. Of 54 necropsies of natural cases in which erysipelas was diagnosed culturally, 46 showed intramuscular or subpleural ecchymotic and suffusion hemorrhages (see fig. 1). In twelve toms the tubular leader or caruncle (flashy tubular appendage on dorsal surface of head) was turgid with an irregular reddish-purple color (see fig. 2). When present, this lesion has appeared to be pathognomonic of this infection. Two birds showed cyanotic, erysipeloid lesions of the skin. One showed endocardial vegetations of the right atrioventricular muscle flap. Two experimentally infected birds which survived for three weeks showed aortic endocardial vegetations.

The liver was often swollen and mottled from congestion, with increased friability and areas of degeneration or anemic infarction. In peracute experimental cases, the liver was slightly swollen and presented a "cooked appearance." This condition was noted in some field cases, both in the liver and in the muscles.

The spleen consistently showed pathologic changes. In most cases it was highly engorged with blood and purplish-black. In other cases it showed diffuse congestion with petechiae and ecchymoses.

Abdominal fat was usually tinged red and petechiated. Ecchymotic and suffusion hemorrhages under the gizzard serosa were noted in many cases, especially in experimentally infected birds. All visceral blood vessels appeared highly injected. An intense enteritis, either catarrhal or sanguino-catarrhal in nature, was noted.

Kidneys and sex organs were congested and frequently showed petechiae or ecchymoses under the serous surface. Sharply outlined rose-colored spots or areas of congestion or hemorrhage were often seen on the pancreas.

Hydropericardium, epicardial and pericardial petechiae and ecchymoses were common. Diffuse skin reddening and a dirty brick-red muscle color were other lesions

noted with varying frequency in the field outbreaks. An occasional bird died showing no lesions other than slight catarrhal enteritis and petechiae on the heart fat.

DIAGNOSIS

Sudden losses of adolescent turkeys in good flesh with septicemic lesions, intramuscular and subpleural ecchymotic and

Fig. 2. Swollen caruncle. This lesion seems to be pathognomonic.

suffusion hemorrhages, and erysipeloid swelling of the caruncle point toward erysipelas, especially if affected birds are mostly males. Beaudette and Hudson[3] first pointed out the diagnostic value of subcutaneous blotch hemorrhage. Madsen[6] noted necrotic changes in the caruncle.

The presence in liver, spleen, or heart-blood smears of clumps and segregated Gram-positive, beaded, slender, pleomorphic rods is further diagnostic evidence of erysipelas (see fig. 3).

Isolation of characteristic, virulent organisms from birds dead of the disease clinches the diagnosis. Protection tests with anti-swine-erysipelas serum in mice further establishes *E. rhusiopathiae* infection as the cause of death.

Isolation of the erysipelas organism from sick birds which are killed and then cultured is not as easy nor as positive as culturing from birds dead of the disease. Bone-marrow culture, as suggested by Hoffman and Hinshaw,[8] is helpful in decomposed specimens.

Cases of hemorrhagic enteritis with subcutaneous blotch hemorrhages and other erysipelas-like lesions have been observed. As blood smears and bacteriologic cultures did not reveal any organism, these conditions have been regarded as not due to erysipelas infection.

DISCUSSION OF EPIZOÖLOGY

Table I summarizes the epizoötiologic data from 16 outbreaks of erysipelas during the past three seasons. Over 30,450 birds were involved, with a mortality of about 1,507 and a slightly higher morbidity. The first three outbreaks occurred in October and November of 1937, and the next five in October, November and December of 1938. During October, November and December of 1939, seven outbreaks occurred. The last outbreak was in February 1940.

The occurrence of the disease in Oregon in the months from October through February agrees with reports from other states. During this season, there is in-

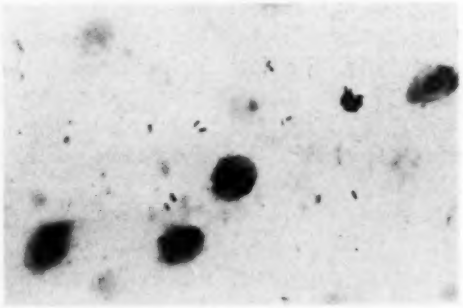


Fig. 3. Photomicrograph of spleen smear from a turkey naturally infected with E. rhusiopathiae (x 785).

creased precipitation and most flocks have matured to the age that fighting among males is common.

Madsen[6] observed that the mortality occurred principally in the males. This fact is emphasized in table I. Nine and six-tenths per cent of the birds lost were females, 82.1 per cent were males, and in 8.3 per cent the sex was undetermined.

The losses strikingly concentrated in the males, and the erysipeloid lesions of the head and neck indicated that infection probably entered through skin abrasions acquired during combat.

In flocks 7, 8 and 13, males and females were on adjacent ranges. There was no re-

TABLE I—Epizoötiologic Data on 16 Flocks of Turkeys Affected with Erysipelas

Flock No.	Total No. Birds	Age (Mo.)	Sex	Approx. Mortality			Other Stock on Range Within 1 Yr.	Approx. Duration (Days)	Management and Control Measures	Effect of Control
				Male	Female	Total				
1	2,300	4½–5½	Mixed			100*	Sheep	20	None	No report
2	600	6	Mixed	25	5	30	Sheep	30	Changed range	Losses subsided
3	1,000	5½	Mixed	60	15	75*	Sheep	30	None	No report
4	5,000	4	Mixed	300	50	350	None	50	Changed range	None
5	2,000	6	Mixed	70	5	75	None	20	Culled and marketed	Salvage†
6	2,000	6	Mixed	47	3	50	Turkeys	30	Changed range, culled and marketed	None
7	500	8	Male	55		55	Sheep	20	Culled and marketed	Salvage
8	500	6½	Male	50		50	Sheep	10	Culled and mktd.; serum	Losses subsided‡
9	4,500	4½	Mixed	460	65	525	None	60	Culled and mktd.; serum	No change
10	1,800	5	Mixed			25*	No report	20	No report	No report
11	900	6	Mixed	13		13	Turkeys	10	Culled and mktd.; serum	Losses subsided‡
12	700	6	Mixed	25		25*	None	10	$MgSO_4$ in water 1 week before; no report	No report
13	2,400	6	Male	114		114	Sheep	15	Culled and marketed	Salvage†
14	3,500	6	Mixed	14	1	15	Turkeys	14	Culled and marketed	Salvage†
15	750	6	Female		1	1	Turkeys	1	None	Loss stopped
16	2,000	8	Mixed	4		4	Sheep	7	None	Loss stopped
Total	30,450			1,237	145	1,507	Sheep 7 Turkeys 4 No stock 4 No report 1	1–60		

*Mortality up to the time birds were brought to laboratory. No further report.
†Loss of grade. Rejects due to poor bleeding.
‡For complete results see Experimental Field Trials.

port of illness or loss among the females during the outbreak in the male flocks. Although females were readily infected experimentally, the low incidence and slow spread of the disease among females in mixed flocks indicated that in hens the infection often did not find a ready portal of entry.

The special Committee on Poultry Diseases of the American Veterinary Medical Association suggested (in 1938) that the source of the infection should be found and pointed out that sheep and fish meal have been suspected. The source of the infection in the outbreaks reported here has not been definitely determined.

The data in table I do not indicate that range previously used by sheep is especially dangerous as a source of erysipelas infection in turkeys, as 8 out of the 16 outbreaks occurred on land unused by sheep for at least 12 months. Furthermore, ovine erysipelothritic polyarthritis has not been demonstrated in the sheep on ranges where outbreaks of erysipelas occurred in turkeys. A summary of seven erysipelas outbreaks in turkeys reported from other states (table II) shows that only three outbreaks occurred on "sheeped-over" range.

Attempts to isolate *E. rhusiopathiae* from sheep bones found on one turkey range (flock 8) were unsuccessful. Turkeys are commonly raised on "sheeped-over" range with no loss from erysipelas. Therefore, the occurrence of outbreaks in turkeys on range previously occupied by sheep may merely be coincidental.

That soil may be a factor in the epizoötic appearance of swine erysipelas has been mentioned by many workers, including Van Es and McGrath,[24] Gay *et al*,[25] Topley and Wilson.[26] Vallee[27] suggested that the pH of the soil exerts an effect on the epizoötic recurrence of swine erysipelas. Van Es and McGrath[24] cited Vallee to the effect that in the regions in which swine erysipelas occurs, the soil contained the germs. Further, Van Es and McGrath stated that "naturally acid soils . . . . destroy the bacilli within the first few days after intro-

[24]Van Es, L., and McGrath, C. B.: Swine erysipelas. Univ. of Nebraska Res. Bul. 84 (1936).

[25]Gay, F. P., and associates: Agents of Disease and Host Resistance (Chas. C. Thomas Publishing Co., Baltimore, 1935), pp. 1048-1053.

[26]Topley, W. W. C., and Wilson, G. S.: The Principles of Bacteriology and Immunity (2nd ed., William Wood & Co., New York, 1937), pp. 284-286.

[27]Vallee, M.: The experiments carried out in France on the acidity of soils and its relation to swine erysipelas. Rev. Path. Comp., xxx (1930), p. 857. Cited editorially in J.A.V.M.A., xcvi (1940), p. 23.

duction." It should be noted that most of the Willamette Valley soils are acid, according to Powers *et al.*[28] Changed soil reaction and soil transfer due to erosion may enter into the ecology of erysipelas infections.

Individual flock histories show many conflicting facts about turkey erysipelas. After the outbreak occurred in flock 4, the birds were moved from a low, flat area of Amity silty clay loam to a partially wooded hill of Melbourne silty clay loam. Losses slacked at first, but flared up at intervals until killing time, nearly two months later.

Catching equipment used in marketing flock 4 was used later to select part of flock 8 for market. After a few days erysipelas appeared among birds in flock 8. Similarly losses in flock 12 occurred a few days after the owner helped a neighbor catch sick turkeys.

The turkeys in flock 9 ranged on a well-drained field of Carleton silty clay loam on which there had been no livestock for several years. Without previous symptoms of illness, 35 birds died the first night of the outbreak. Changing range to the hill of Melbourne silty clay loam used the year before by flock 4, segregating sick birds, and administering serum failed to control the disease.

Birds in flock 15 were reared on the Amity silty clay loam range used the year before by flock 4. There was no abnormal mortality during the rearing period in flock 15 despite the fact that the same feed and water trucks were used and the same attendants cared for flocks 9 and 15 throughout the season. After breeder hens were selected and moved to a new range, the balance of flock 15 was sold. One week later one breeder hen was found dead of erysipelas infection. No further loss from erysipelas was reported.

Seven hundred and fifty breeder hens selected from flock 9 and a like number from flock 15 were placed on the same range after most of the birds were marketed. No losses due to erysipelas were reported during the following breeding season.

Similarly, erysipelas was not carried from flock 14 to a younger group of turkeys, although the trucks and the attendants were common for both flocks.

Recurrence of erysipelas infection in turkeys on the same range in subsequent years has not been observed.

In brief, aside from the occurrence of the disease among the males of a flock in

[28] Powers, W. L., Jones, J. S., and Ruzek, C. V.: Composition, rating and conservation of Willamette Valley soils. Oregon Agr. Exp. Sta., Bul. 365 (1939).

TABLE II—Epizoötiologic Data of Erysipelas in Turkeys Reported from Seven States

Reported by	Place and Time	Total No. Birds	Age (Mo.)	Sex	Approx. Mortality	Other Stock on Range Within 1 Yr.	Approx. Duration (Days)	Management and Control Measures	Effect of Control
Beaudette and Hudson	N. J. 11–34	500	4		200	Sheep (few) Misc. farm animals*	10	Crowded quarters; wormed 4 days before	Spontaneous recovery
Madsen	Utah 11–36	1,300	5½	Mixed	300 ♂ 25 ♀	Sheep	30	None reported	Losses subsided†
	Mass. 10–36	120	5	Mixed	12	Miscellaneous Cow	14	Wire porches; serum and free range	Losses subsided
Van Roekel, Bullis and Clarke	Vt. 10–36	10,000	4–5	Mixed	300	Cattle, wild life, hogs*	35	Slaughter,§ flush and change range	Losses continued but subsided‡
	N. Y. 11–36	1,900	4–6	Mixed	60‖	None	28	Moved to shelter; slaughter	Losses subsided
Hoffman and Hinshaw	Calif. 11–37	2,500	6	Mixed	90 ♂ 0 ♀	Sheep	35	Changed range	Losses subsided
Schlotthauer and Thompson	Minn. 11–38	100	6	Mixed	35	Swine*‡		Wire floor	No report

*Swine and turkeys had no contact.
†Many sick birds recovered. 1,700 birds on land not washed over by sheep corral contents remained well.
‡Hogs eating droppings or dead birds were not infected.
§Some slaughtered birds did not dress well.
‖Losses confined to portion of birds on range without shelter.

the fall of the year, the epizoölogy was unpredictable. Losses usually occurred just prior to marketing. Even in birds saved from infected flocks for breeding purposes, losses subsided after the meat birds were marketed. Only once has erysipelas occurred in a breeding flock (flock 16).

Waller[5] has pointed to the possibility of game birds as carriers of infection. Van Roekel *et al*[7] mentioned garbage. Beaudette and Hudson,[3] Madsen,[6] Hoffman and Hinshaw,[8] Beaudette[29] and Hinshaw[30] suspected sheep as infection reservoirs. Attention has also been drawn to fish meal as a possible source. There is little evidence to support any contention as to the source of the organism.

Soil seems the most logical reservoir, since *E. rhusiopathiae* may be a soil saprophyte and is said by many to change very readily from saprophyte to pathogen. This apparently altered virulence has not been explained. In field outbreaks it may depend on lowered resistance of the hosts.

Certain phenomena noted in immunization and transmission work indicated that successive turkey passages raised virulence, since birds succumbed to smaller and smaller doses of virulent culture despite the use of antiserum. Possibly such passages have occurred during natural outbreaks.

Ten Broeck,[31] before septicemic erysipelas was clinically important in the United States, suggested that *E. rhusiopathiae* may actually be a secondary invader, while Smith[32] long ago indicated that diet was of

[29]Beaudette, F. R.: Swine erysipelas in turkeys. Hints to Poultrymen, New Jersey Agr. Exp. Sta., xxvi (1939), No. 4.

[30]Hinshaw, W. R.: Diseases of turkeys in the United States. Proc. Seventh World's Poultry Cong. & Expos. (1939), p. 237.

[31]Ten Broeck, C.: Studies on *Bacillus muriseptic-us*, or the Rotlauf bacillus isolated from swine in the United States. J. Exp. Med., xxxii (1920), pp. 331-343.

[32]Smith, T.: Swine erysipelas or mouse-septicemia bacilli from an outbreak of swine disease. U. S. Dept. Agr., Bur. Ani. Ind., 12th and 13th Ann. Rpts. (1897), pp. 166-174.

TABLE III—Experiments with Anti-Swine-Erysipelas Serum in Turkeys

Trial	Group	Sex ♂	Sex ♀	Culture Time	Culture Site	Culture Amt. per Bird	Serum Time	Serum Mode	Serum Amt. per Bird	Results Lived	Results Died*	Notes
						(cc.)			(cc.)			
I	A	2		12- 2-38	4 cc., Ip.	6 37	12- 2-38	Im.	1	2		Survivors proved refractory to inoculation with virulent culture 67,167,215 days after first inoculation
	B	2		12- 2-38	2 cc., Im.	6 37	12- 2-38	Im.	5	1	1	
	C	2		12- 2-38	0 25 cc., Eye	6 37	12- 2-38	—	0		2	
	D	1		12- 2-38	0 12 cc., Nose	6 37	12- 2-38	—	0	1†		
II	E	2	2	2- 7-39	caruncle	1	2- 7-29	Im.	2	2 ♀	2 ♂	One survivor resisted reinoculations with virulent culture after 100 and 222 days, one after 222 days, and 3 after 368 days. One bird was not tested for immunity
	F	2	2	2- 7-39	caruncle	1	2- 7-39	Im.	4	3	1 ♀	
	G	2		2- 7-39	caruncle	1	2- 9-39	Im.	4	1	1	
	H	2	2	2- 7-39	caruncle	1	2- 7-39	—	0		4	
III	I		2	5- 4-39	caruncle	1	5- 4-39	Im.	4	1	1	Survivor resisted a lethal dose of virulent culture 122 days after first inoculation
	J		2	5- 4-39	caruncle	1	5- 5-39	Im.	4		2	
	K		2	5- 4-39	caruncle	1	5- 4-39	—	0		2	
IV	L		2	5-18-39	caruncle	0 25	5-18-39	Im.	4	1	1	Survivor resisted a lethal dose of virulent culture 108 and 382 days later
	M	1	1	5-18-39	caruncle	0 25	5-19-39	Im.	4		2	
	N		2	5-18-39	caruncle	0 25	5-18-39	—	0		2	
V	O	2		11-17-39	caruncle	0 25	11-15-39	Im.	2		2	Birds inoculated with culture 11-17-39 showed no reaction and hence were reinoculated five days later
				11-22-39	caruncle	0 50						
	P	2		11-17-39	caruncle	0 25	11-15-39	Im.	4		2	
				11-22-39	caruncle	0 50						
	Q	2		11-17-39	caruncle	0 25	11-17-39	Im.	4	2		
				11-22-39	caruncle	0 50						
	R		2	11-17-39	wing web	0 25	11-15-39	—	0		2	In group R, one bird injected into wing web, one into caruncle
				11-22-39	caruncle	0 50						

Im. = Intramuscularly into pectoral muscles. Ip. = Intraperitoneally.
*Characteristic cultures of *E. rhusiopathiae* were isolated from each bird that died in these experiments.
†Survived previous injection of avirulent *E. rhusiopathiae*.

etiologic importance. Stiles and Davis[33] observed that the incidence of the disease increased after rainstorms.

Van Es and McGrath[24] cited Nocard and Leclainche (1903) as saying, ". . . . at the present state of our knowledge, it is impossible to explain the mysterious behavior of the contagion." Unfortunately, this is still true.

EXPERIMENTAL USE OF ANTI-SWINE-ERYSIPELAS SERUM

Van Roekel *et al*[7] first mentioned the apparently successful use of commercial antiserum in controlling outbreaks of turkey erysipelas, but considered the number of controls insufficient to allow proper evaluation of the benefits. Vianello[34] reported successful control of an outbreak of erysipelas in pheasants (after the loss of 80 birds) by the use of antiserum. Graham *et al*[4] stated that, "Attempts to protect ducklings (with erysipelas serum and bacterins) . . . . were not successful."

There is little information regarding the efficacy of various methods of preventing and controlling erysipelas in turkeys. A series of laboratory tests were conducted to ascertain the value of commercial anti-swine-erysipelas serum. The results of these trials are presented in table III.

Turkeys used in these trials were reared at the laboratory in clean metal cages equipped with rod floors. Feed and water were available, through a grating, on the outside of the cage at a height that would not permit contamination with droppings. During the trials two birds were kept in each cage.

Commercial anti-swine-erysipelas serum, prepared from hyperimmune horses, was injected into the pectoral muscles of the turkeys. Autopsy of some birds within 48 hours following injection indicated that it was absorbed by that time.

All cultures used were checked for viability and purity and for pathogenicity toward mice (*M. musculus*) before use.

Cultures used in trial I were pooled isolations from three field cases of erysipelas. In this trial a large amount of broth culture was administered in four different locations in each bird, as noted in table III, to insure infection. In trial II a culture isolated from a bird that died in trial I was used. In each trial a culture isolated from a bird that died in the previous trial was used. Hence, the culture used in trial V had undergone four turkey passages. Fatalities resulting from increasingly smaller dosages of culture indicate that this turkey passage markedly increased the virulence. The average time between injection and death decreased, and antiserum was less effective in the later trials.

Except in trial I, broth cultures have been inoculated into the caruncle of toms and, where present, of hens. In other hens the cultures were injected subcutaneously over the skull. Typical erysipeloid caruncles were produced which returned to normal within four to six weeks in surviving birds.

In birds that died in these trials, death occurred within five days after artificial infection, except in two birds. One control in trial I and one protected bird in trial II died with vegetative endocarditis after 21 days. Typical cultures of *E. rhusiopathiae*

TABLE IV—Field Trials with Anti-Swine-Erysipelas Serum for Turkeys

Field Trial	Dosage and Mode of Administration	Recovered		Died	
		Male	Female	Male	Female
I*	2 cc., Im.	28	1	6	0
	No treatment	13	0	7	0
II†	2 cc., Im. 2 cc., Iv.	61	25	23	11
	4 cc., Im.	61	25	25	6
		122	50	48	17
	No treatment	63	26	21	7
Totals Trial I & II	Treated	150	51	54	17
	Controls	76	26	28	7

*Flock 8 in table I.
†Flock 9 in table I.
Im. = Intramuscularly.
Iv. = Intravenously.

[33] Stiles, G. W., and Davis, C. L.: Swine erysipelas and its economic importance. J.A.V.M.A., lxxxv (1934), pp. 895-906.

[34] Vianello, G.: *Erysipelothrix rhusiopathiae* infection in pheasants. (Title trans.) Clin. vet., Milano, lxi (1938), pp. 234-243. Abst., Vet. Bul., ix, p. 69.

were isolated from all birds dying in these trials.

In trial I, birds were bled, temperatures recorded and blood films stained daily. Pre-inoculation temperatures ranged between 105.5° and 106.8° F. The greatest rise in temperature in any one bird was 3.2° 44 hours after inoculation. At that time all birds but one moribund turkey showed the greatest pyrexia. Forty-four hours after injection of broth cultures, typical Gram-positive bacteria were noted in blood films from each of the six birds. In one they were demonstrated after 24 hours.

Work already reported by Schoening *et al*,[35] Van Roekel *et al*[7] and the writers[9] indicates the rapid appearance of demonstrable agglutinins following injection of antiserum and bacteria.

Blood samples were drawn from the birds in trial I daily for eight days, every second day for ten more days and then at weekly intervals for five weeks. Two days after injection, plate antigen and a dilution corresponding to 1:50 being used, a slight agglutination reaction was observed in three birds. On the third day, all four survivors exhibited definite agglutination reactions at this dilution. The agglutination titer increased daily until the eighth day, after which the titer held nearly constant for ten days. It then gradually decreased so that no agglutinins could be demonstrated in the final blood samples that were drawn. No positive reactions were observed in dilutions higher than 1:200.

Birds were weighed at the time of inoculation in tests I, II, III and IV. Daily weights were recorded in the latter three trials. No bird surviving the inoculation of culture lost more than 1⅔ lb. during the week following exposure. With the exception of one turkey which died with endocardial vegetations three weeks after exposure to virulent culture, any weight lost was more than regained within three weeks.

To check its therapeutic efficacy, birds in groups G, J and M in trials II, III and IV, respectively (table III), were administered antiserum 24 or 48 hours after injection with culture. In trials III and IV, especially, the damage done by the organism was evidently too grave before the antiserum had a chance to prove beneficial.

In trial V an inexplicable variation in virulence of the organism was encountered. As usual, a strain of the organism isolated from a dead bird in the previous trial was used. This was transferred to difco tryptose phosphate broth. Three days later more of the same broth was inoculated from the same source of inoculum. Both broth cultures grew well and proved lethal to mice. The second broth culture was used for inoculation in trial V on November 17, 1939. No effects were noted from this inoculation. Equal amounts of the first and second broth cultures were pooled and inoculated into the birds five days later. Typical erysipelas infection resulted.

The results in groups P and Q of trial V are noteworthy. The birds in group P were injected with antiserum seven days before receiving virulent culture which killed them. Those in group Q, protected only five days before, survived. This seemingly short period of immunity conferred by antiserum has been further emphasized by field observations in flock 9. Koves and Hoffman[36] stated that heterologous antiserum confers a much more transitory immunity than homologous serum.

Antiserum was obtained by bleeding three toms previously hyperimmunized to *E. rhusiopathiae* by massive doses of virulent culture. Mice given a dose of the serum, equal in amount to a protective dose of commercial antiserum, succumbed to an injection of virulent culture. However, four times this volume of antiserum from turkeys protected mice against lethal doses of virulent culture.

Of 28 turkeys inoculated with from 1 to 5 cc. of commercial antiserum, only 13 survived lethal doses of virulent *E. rhusiopathiae* and were subsequently proved re-

[35]Schoening, H. W., and Creech, G. T.: Swine erysipelas: studies on Erysipelothrix with particular reference to serological diagnosis. J.A.V.M.A., lxxxviii (1936), p. 310.

[36]Koves, J., and Hoffman, F.: The duration of retention of foreign antiserum injected into domestic animals. (Title trans.) Kozl. Oesszehas. elet-es kortan Korebol., xxviii (1937), pp. 139-144. Abst., Vet. Bul., ix, p. 559.

sistant to further inoculations of cultures. Twelve control birds unprotected by antiserum died. One previously protected bird lived.

This indicated that antiserum was moderately effective in controlling artificial infection. The duration of protection afforded by inoculation of antiserum seemed extremely short.

SAPONIN VACCINE AND SULFANILAMIDE

Due to relatively low efficiency of antiserum, experiments with twelve birds were set up to test the value of glucoside vaccine similar to anthrax vaccine of Mazzucchi.[37] Radvilla[38, 39] reported that saponin vaccines in erysipelas were "not good," but his reports were not noted until after the saponin work was started.

Two Gm. of saponin per 100 cc. of medium was added to difco tryptose phosphate broth and sterilized. The same culture of organisms used in trial V (table III) was inoculated into the 2 per cent saponified broth, incubated 48 hours, then placed at room temperature in the light. Growth appeared more profuse than in difco tryptose phosphate broth without saponin.

Inoculation of sterile 2 per cent saponified broth into turkeys caused a long-standing edema with slight local necrosis, but no permanent damage.

One-half cc. of saponified culture of *E. rhusiopathiae* 60 days old was inoculated into each of three 6-month-old turkeys at the time trial V was started. One bird was inoculated into the web of the wing, one subcutaneously beneath the pygostyle, and one into the caruncle. The bird injected with saponin vaccine into the wing web died after 92 hours. Pure cultures of *E. rhusiopathiae* were recovered at autopsy.

The two other birds survived and showed only local edema at the point of injection. Eighty-seven days later they withstood a lethal dose of culture which killed an unprotected control.

Similar vaccine was made up by inoculating heart blood of a bird dead of erysipelas into 2 per cent saponified difco tryptose phosphate broth. After incubation, this lot of saponin vaccine was checked for purity and stored in the light at room temperature.

Fifty-nine days later 0.5 cc. of the second lot of saponin vaccine was injected into the wattle of each of two turkeys and .25 cc. injected into two others. All four died within 46 to 88 hours. Two birds injected with unsaponified difco tryptose phosphate broth culture died 64 and 116 hours later. One survivor of the first saponified vaccine trial withstood 3.5 cc. of this culture. Pure cultures of *E. rhusiopathiae* were recovered from the six birds. This saponified vaccine when 115 days old was injected into the caruncle of a tom (0.3 cc.) and a hen (.25 cc.). Both birds died within five days.

Only two out of nine birds inoculated with saponin vaccine survived the inoculation and were subsequently proved resistant to lethal doses of virulent cultures of *E. rhusiopathiae*. This indicates that saponin vaccines have no practical value.

Two birds were injected in the caruncle with 1 cc. of virulent culture at the time trial III was started. Treatment with sulfanilamide was begun 30 hours later with an initial oral dose of 5 gr. per bird. One bird died 38 hours after injection. The remaining bird was given 10 gr. of sulfanilamide at 39 and 45 hours, but was dead 55 hours after the injection.

Culture filtrates of *E. rhusiopathiae* tested on mice had no protective value.

FIELD TRIALS

When flock 8 (table I) became sick with erysipelas, opportunity was afforded to test the value of commercial anti-swine-erysipelas serum. Arrangements were made to cull the flock, to sell the well birds, and to treat the sick birds.

Droopy birds were segregated and banded. Two out of every three birds were

[37] Mazzucchi, M.: Le charbon hematique (anthrax). Proc. 12th Int. Vet. Cong., ii (1934), pp. 138-156.

[38] Radvilla, P.: Immunization against swine erysipelas with glucoside cultures. (Title trans.) Wein. Tierärztl. Monatsch., xxiv (1936), pp. 603-604. Abst., Vet. Bul., ix, p. 452.

[39] *Ibid.:* Vaccination against swine erysipelas by glucoside vaccination. (Title trans.) Vet. ir Zootech., Kovno., xiv (1937), pp. 257-267 and 289-300. Abst., Vet. Bul., ix, p. 682.

inoculated into the pectoral muscles with 2 cc. each of commercial anti-swine-erysipelas serum. Every third bird was left as an untreated control. Condition and degree of droopiness of each bird were noted and a record kept of the subsequent disposal of the birds. All but one bird, dying after this field trial started, were autopsied and yielded typical *E. rhusiopathiae*. The results are given as field trial I in table IV.

Forty-three per cent of treated birds and 40 per cent of untreated controls were classed as very droopy. Seventeen per cent of the treated birds and 35 per cent of the untreated controls died.

These results suggested that commercial antiserum might be a worthwhile aid in controlling turkey erysipelas.

Nearly a year later when erysipelas occurred in flock 9 (table I), it was possible to again test the value of commercial anti-swine-erysipelas serum in a field outbreak of the disease. This trial is shown as field trial II in table IV. It was run on sick birds segregated from flock 9 between October 23 and December 21, 1939. Losses at that time were increasing, and as the birds were ready for market, slaughter was advocated.

Segregated sick birds were banded numerically and were divided in three groups as indicated in table IV. Birds were treated in order; one kept as a control, and then two birds treated with serum. Records were kept of the lesions, the degree of droopiness, and other information.

Of 117 birds injected with 4 cc. of antiserum intramuscularly, seven (5.9 per cent) were very droopy, 100 moderately droopy and ten either normal or culls. Twelve (10 per cent) birds were very droopy in the other treated group of 120 and 97 were moderately so. Ninety-six control birds out of 117 were moderately droopy while eight (6.8 per cent) were very droopy. Eight per cent of all the treated birds were very droopy. Five birds were nearly moribund in the treated groups and only one control was extremely sick.

Nine necropsies of banded birds were made during the outbreak. *E. rhusiopathiae* was consistently isolated. Twelve unbanded birds from the main flock autopsied at various times during the outbreak yielded characteristic cultures of *E. rhusiopathiae*.

In one treated group (4 cc. intramuscularly), 29 per cent of the males, 19.35 per cent of the females, or 26.5 per cent of both sexes, died. Twenty-seven and three tenths per cent of males, 30.5 per cent of the females, or 28.3 per cent of both sexes, died in the other treated group (2 cc. intravenously, 2 cc. intramuscularly). Twenty-seven and four tenths per cent of all treated birds died.

Among the controls, 25 per cent of the males, 21.2 per cent of the females, or 23.8 per cent of both sexes, died. This indicates a survival rate among controls higher than among treated birds, but it should be remembered that there were more moribund birds in the treated groups. The mortality of the different groups and sexes did not seem to vary significantly.

Moreover, twelve of the birds died more than ten days after serum injection, emphasizing the short duration of serum protection. Five of the twelve had swollen caruncles at the time of injection. Two of these received 8 cc. of serum, were very ill, but recovered and were returned to the main flock. About a week later they died. This illustrates that using serum for passively protecting the birds and depending on field inoculation for nonlethal infection to produce a solid active immunity is both uncertain and unsatisfactory.

Field trial II indicates that because of uncertain and short-lived protection, as well as expense, anti-swine-erysipelas serum had no value in the control of turkey erysipelas.

Summarizing both field trial I and field trial II, 26.1 per cent of the antiserum-treated birds and 25.5 per cent of the untreated controls died.

Four hundred and fifty of about 900 birds remained in flock 11 at the time erysipelas was diagnosed, as early prime birds had been sold. Thirteen birds had been lost prior to that time. The hens and all apparently well toms were marketed. Four cc. of commercial anti-swine-erysipelas serum was injected into the leg of each of

110 remaining males by Dr. E. W. Coon of Forest Grove, Ore. No further losses occurred and the 110 males were marketed 26 days later. As no untreated controls were left, it is difficult to evaluate how much the administration of antiserum altered the course of the disease.

The cost of antiserum would be about 5 cents per injection per bird plus the expense of handling the turkeys. Results noted both in the field and laboratory indicate that the value of such antiserum injection is questionable.

Because of short duration of protection, the uncertain results and the expense both of serum and handling the birds, serum immunization of an entire well flock does not seem economically justifiable.

SUMMARY

1) Data presented on 16 outbreaks of infection with *Erysipelothrix rhusiopathiae* occurring in turkeys during a three-year period indicate that the epizoölogy of this infection in turkeys is highly erratic.

2) The outbreaks occurred during the months of October, November and December, with one exception.

3) The vast majority of the birds affected were males (82.1 per cent).

4) The most pathognomonic lesion was a turgid, reddish-purple caruncle.

5) The lesions most consistently encountered were intramuscular and subpleural ecchymotic and suffusion hemorrhages. These lesions, however, were not confined exclusively to this infection.

6) Diagnosis of erysipelas depended on isolation and identification of the causative organism.

7) Laboratory and field trials indicate that commercial anti-swine-erysipelas serum has no practical value as a treatment or prophylactic in the control of erysipelas infection in turkeys.

8) Segregation of sick birds and slaughter of well birds seemed to be the most economical method of handling these outbreaks.

ACKNOWLEDGMENT

Acknowledgment is due Dr. H. W. Schoening, Pathological Division, United States Bureau of Animal Industry, for supplying antigen for use in these studies.

Macroscopic Differentiation of Lesions of Histomoniasis and Trichomoniasis in Turkeys*

ENA A. ALLEN, Ph.D.

Beltsville, Md.

DURING the past several years routine microscopic examinations of smears from so-called blackhead lesions from livers of turkeys sent in for diagnosis frequently showed numerous trichomonads and few or no histomonads. Since the turkeys had been dead for several hours before examination, it was not possible to determine whether the trichomonads present represented primary infections. Later, however, trichomonads were frequently found alone or associated with histomonads in cecal and liver lesions from turkeys examined within an hour or two after death. Observations have shown that the lesions containing only trichomonads were characteristically different from those in which only histomonads were present, although they were superficially similar enough to be confused upon casual study. Subsequent studies have indicated that while the trichomonads may occur in lesions containing no histomonads, the lesions frequently contained the two parasites together. Such lesions could be differentiated macroscopically from those of pure infections of either of the two protozoan parasites.

The finding of trichomonads in pure infection in characteristic lesions of naturally infected birds suggested that these organisms might be pathogenic. Success in obtaining and maintaining bacteria-free cultures of the trichomonads made possible the experimental infection of susceptible turkey poults to test this possibility. A sufficient number of experimentally infected birds developed the characteristic cecal and liver lesions to warrant the conclusion that trichomonads are capable of producing a frequent and sometimes fatal enterohepatitis independent of *Histomonas meleagridis*. In such cases trichomonads were recovered from time to time in pure, bacteria-free cultures from liver lesions.

The trichomonads in question were tentatively determined by the author[1] as Pentatrichomonas species in 1936. However, a further morphologic study[2] has shown that the above species is identical with *Trichomonas gallinarum*, described by Martin and Robertson[3] from the ceca of the chicken and turkey. As the experimental evidence collected by the writer since 1936 strongly indicates that *T. gallinarum* is capable of producing an enterohepatitis, with lesions that may occur alone or in combination with those of histomoniasis, it is important from the standpoint of accurate diagnosis to distinguish between these two conditions. The observations reported in this paper are confined to the macroscopic differentiation of the cecal and liver lesions in pure and mixed infections of *T. gallinarum* and *H. meleagridis*, since the lesions constitute the most obvious postmortem evidence of these infections.

MATERIALS AND METHODS

Material for experimental infections was obtained by culturing *T. gallinarum* on a modified Boeck and Drbohlav's[4] medium which consisted of egg slants covered with Locke's solution to which small amounts of liver extract and defibrinated turkey blood were added. The trichomonad was obtained seven times in bac-

*From the Zoölogical Division, Bureau of Animal Industry, U. S. Department of Agriculture. This investigation was carried out at the Beltsville Research Center, Beltsville, Md.

[2]Allen, Ena A.: A redescription of *Trichomonas gallinarum* Martin and Robertson, 1911, from the chicken and turkey. Proc. Helminth. Soc., vii (1940), p. 65.

[1]Allen, Ena A.: A Pentatrichomonas associated with certain cases of enterohepatitis or "blackhead" of poultry. Trans. Amer. Mic. Soc., lv (1936), p. 315.

[3]Martin, C. H., and Robertson, Muriel: Further observations on cecal parasites of fowls with some reference to the rectal fauna of other vertebrates. Part I. Quart. J. Micros. Sci., lvii (1911), p. 53.

[4]Boeck, W. C., and Drbohlav, J.: The cultivation of *Endamoeba histolytica*. Amer. J. Hyg. v (1925), pp. 371-407, pls. 1-4.

teria-free cultures from characteristic liver lesions of turkeys soon after death from trichomoniasis. In five of these instances the material came from naturally infected birds, and in two from experimentally infected turkeys. *H. meleagridis*, which was obtained from characteristic histomonad liver lesions, was also cultivated on a modified Boeck and Drbohlav's medium to which sterile rice starch was added; however, the material was not bacteria free. All cultures were incubated at 41.5° C. and transplants were made every three or four days.

In order to produce experimentally typical lesions of trichomoniasis, cultured trichomonads were inoculated orally into 75 7-day-old poults. Forty-eight other uninoculated birds were held as controls. The inoculated birds and controls were kept indoors on wire floors until 6 weeks old. The pens and all equipment were thoroughly cleaned daily to minimize the possibility of accidental infection. At the age of 6 weeks, the poults were removed to outside runs which had not been occupied by livestock of any kind during the previous two years. Typical lesions of histomoniasis for comparison with those of trichomoniasis were obtained by experimentally inoculating 26 poults with *H. meleagridis* obtained from cultures. As yet no attempt has been made to produce experimentally mixed infections of trichomonads and histomonads.

Livers showing the characteristic macroscopic lesions of trichomoniasis and histomoniasis produced by experimental infections and of mixed infections acquired under natural conditions were preserved according to the method of Kaiserling[5] and later photographed.

RESULTS OF INFECTIONS

Ten of the 75 birds inoculated with *T. gallinarum* developed trichomoniasis between the ages of 3 and 7 months. Six of the infected birds died from the infection and showed typical cecal and liver lesions *post mortem*. The four other infected birds were positively diagnosed by observing the lesions through incisions in the body wall, according to the method described by Olsen and Allen.[6] The remaining 65 birds have been held for further observations.

Two of the 48 control birds died at about 3 months of age and showed cecal but no liver lesions of trichomoniasis *post mortem;* another bird died at 4 months of age from histomoniasis.

Twenty-four of the 26 poults inoculated with *H. meleagridis* died of acute histomoniasis.

MACROSCOPIC APPEARANCE OF LESIONS

Lesions of Trichomoniasis.—Liver lesions produced by *T. gallinarum* (fig. 1) appear as granular, cream-colored areas of necrosis with irregular outlines. These areas are level with or elevated above the surface of the liver. Individual lesions

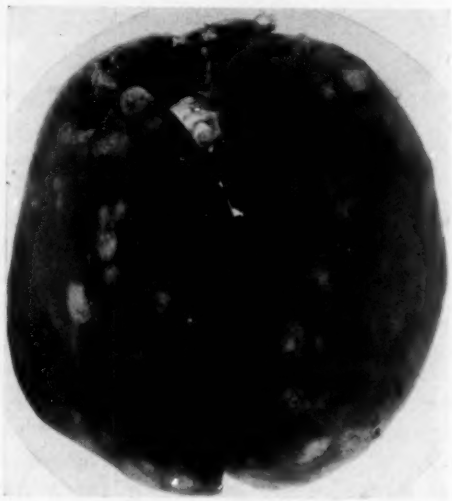


Fig. 1. Turkey liver, showing typical lesions of T. gallinarum.

range in size from small, indistinctly defined areas no larger than a pin point to relatively large, clearly defined areas having a diameter of about three-fourths of an inch. They appear to be composed of numerous minute, rice-shaped granules which radiate from the center in a more or less stellate formation. The fully developed lesions, which appear as cheesy masses composed of closely packed granules, are distinctly elevated above the surface of the liver. They are quite distinct in appearance from the circular, depressed lesions of histomoniasis. The cecal lesions are similar to those developed in the liver, but are less distinctly outlined.

Birds experimentally infected with *T.*

[5] Kaiserling, C.: Weitere Mittheilungen über die Herstellung möglichst naturgetreuer Sammlungspräparate. Virchow Arch., cxlvii (1897), pp. 389-417.

[6] Olsen, Marlow W., and Allen, Ena A.: The treatment of cecal and liver trichomoniasis in turkeys by fever therapy. (In press.)

gallinarum sometimes develop the acute type of infection, characterized by diarrhea and occasional mortality in the young turkeys a few days after inoculation, but more often they develop the chronic type, the symptoms of which do not appear for several weeks after infection. Cecal or liver lesions, which are apparently associated with the chronic type of the infection,

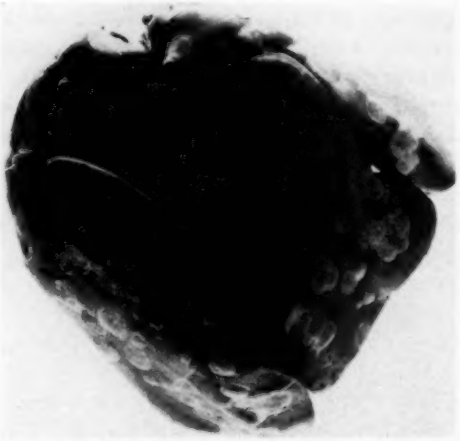


Fig. 2. Turkey liver, showing typical lesions of H. meleagridis.

have not been observed in experimentally or naturally infected birds under 10 to 12 weeks of age. These lesions have been observed frequently in young birds dying from histomoniasis. Thus, observations on birds infected experimentally with both *T. gallinarum* and *H. meleagridis* have shown that chronicity is more common in trichomoniasis than in histomoniasis. The ceca from birds infected with *T. gallinarum* closely resemble those from turkeys infected with *H. meleagridis* in that they contain cheesy cores of blood-stained tissue debris.

Lesions of Histomoniasis.—The liver lesions (fig. 2) that develop following experimental infection of turkeys with *H. meleagridis* are from seven-sixteenths to one inch in diameter. They are more or less circular, cream-colored, and slightly depressed below the surface of the liver. The borders of the lesion are narrow, but well defined, and enclose web-like, radiating formations of necrotic material. In heavy infections the liver lesions sometimes become confluent to form large necrotic areas on the surface of the liver. Cecal lesions in cases of histomoniasis closely resemble those found on the liver, but are less pronounced and tend to ulcerate.

The above description of lesions of histomoniasis as given by the present writer agrees very closely with that given by Smith[7] of the type of lesion occurring in naturally infected birds. As in trichomoniasis, a core composed of a cheesy material usually mixed with blood may be present in one or both ceca.

Liver lesions occur in turkeys as soon as two or three weeks after experimental infection with *H. meleagridis.*

Lesions of Mixed Infections.—Liver lesions (fig. 3) produced by *T. gallinarum* and *H. meleagridis* associated in the same area are large, circular, markedly depressed areas with thick, slightly elevated

Fig. 3. Turkey liver, showing lesions of a mixed infection of T. gallinarum and H. meleagridis.

granular borders. In some instances cream-colored granules are sparsely scattered throughout the central region as well as in the outer edge of the lesion. Associated with this type of lesion may be found a few individual necrotic areas similar in appearance to those found in birds that

[7]Smith, T.: Further investigations into the etiology of the protozoan disease of turkeys known as blackhead, enterohepatitis, typhlitis, etc. J. Med. Res., xxxiii (1915), p. 243.

have been inoculated with pure cultures of either *T. gallinarum* or *H. meleagridis*.

Lesions resulting from mixed infections with *T. gallinarum* and *H. meleagridis* have never been observed *post mortem* in very young birds. It is probable that in the past these lesions have been often diagnosed as purely histomonad lesions, since the trichomonads are not easily demonstrated by the smear method. A positive diagnosis depends upon their presence in cultures.

Menzani[8] has given an illustration of a blackhead liver which shows typical *T. gallinarum* lesions alone and in combination with those of *H. meleagridis*. That writer stated that the turkey from which this liver was taken had a mixed infection of Blastocystis and Trichomonas in the ceca. He apparently confused *H. meleagridis* with Trichomonas and was unable to demonstrate trichomonads in the liver.

[8]Menzani, Cesare: Osservazioni e ricerche su l'enteroepatite infettiva dei tacchini. Rivista "La Clinica Veterinaria" (Nov. 1933), p. 3.

SUMMARY

Three types of liver lesions found in cases of enterohepatitis in turkeys are differentiated as follows: Liver lesions resulting from pure infections of *Trichomonas gallinarum* are irregular in outline, granular in appearance, and are level with or slightly elevated above the surface of the liver. Lesions produced by pure infections of *Histomonas meleagridis* are more or less circular, have narrow, distinct borders enclosing web-like formations of necrotic material, and are slightly depressed below the surface of the liver. Lesions in livers of turkeys having natural mixed infections of *T. gallinarum* and *H. meleagridis* are relatively large, circular, markedly depressed areas, with slightly raised granular borders and sometimes with granular centers. Since under natural conditions these infections may occur separately or combined, an accurate diagnosis based on differentiation of lesions is important.

Studies of Chick-Embryo-Propagated Equine Encephalomyelitis Virus and Vaccine: Antigenicity and Preservation*

M. S. SHAHAN, D.V.M., and E. A. EICHHORN, V.M.D.

Washington, D. C.

FOLLOWING the demonstration by Higbie and Howitt[1] of the multiplication of equine encephalomyelitis virus planted in chick embryos, and subsequent to the development of formolized vaccine from such tissue by Beard, Finkelstein, Sealy and Wyckoff,[2] it seemed desirable to determine what effect continuous passage in embryos might have on the antigenic properties of the virus.

A strain of virus derived from the brain of an Iowa horse in 1937 was used in these studies. The virus had been identified as type-specific western strain following cross-immunity tests[3] in guinea pigs.

A suspension of brain tissue derived from the seventh intracerebral passage of the virus in guinea pigs was used for the primary injection of the embryos, which varied in age from 9 to 12 days throughout these studies, but they were usually 11 days old. The virus was carried through 22 serial passages in embryos, after which propagation was carried out in two series, one designated "C," continuing from embryo to embryo through a total of 226 serial passages; the other designated "I," also carried in series in embryos but periodically interrupted by an intracerebral inoculation of the primary host, a horse, from the brain of which virus was recovered through intracerebral injection of guinea pigs for return to embryo passages in series. In brief, the "I" series consisted of the following, in order: 22 passages in embryos, one passage through a horse, one passage through guinea pigs, 36 passages in embryos (I-1), one passage in the horse, one passage in guinea pigs, 22 passages in embryos (I-2), one passage in the horse, one passage in guinea pigs, finally terminating in the succeeding 35th passage in embryos (I-3). Transfers from embryo to embryo were made generally at one- to 10-day intervals, 10^{-1} to 10^{-4} dilutions of embryo tissue or undiluted allantoic fluid being used for the seed virus.

At intervals in both described series, titrations of the virus content of the embryo tissue were carried out by intracerebral injection of guinea pigs and/or Swiss mice. The former varied considerably in size and weight without apparent significant difference in susceptibility, but it was early observed that weanling mice of certain inbred strains were more uniformly susceptible than mature or outbred mice. A primary dilution of 10^{-1} was prepared by adding 1 cc. of finely divided embryo tissue (ground in a glass Ten Broeck tissue grinder or a colloidal mill) to 9 cc. of physiological salt solution. After the primary suspension was thoroughly mixed, it was allowed to settle for one-half to two hours in the refrigerator. The final serial ten-fold dilutions were made in chilled physiological salt solution shortly before injection. Guinea pigs (two with each dilution) received 0.1 cc. and mice (two to four with each dilution) .03 cc., and usually, when the two test species were run at the same time, the minimum lethal dilution was the same for both. The end dilution varied from time to time from 10^{-5} to 10^{-8} which was rarely exceeded.

In all, 17 separate vaccines were prepared at intervals from embryos in the two series, including one from near the end of each. The vaccines consisted of 20 per cent suspensions in physiological salt solution, the whole being formolized to 0.4 per cent. After determining through intracerebral injection of vaccine in guinea pigs that the virus had been inactivated by the formalin,

---

*From the Bureau of Animal Industry, U. S. Department of Agriculture. Presented before the Section on Research at the 77th annual meeting of the American Veterinary Medical Association, Washington, D. C., August 26-30, 1940.

[1]Higbie, Elizabeth, and Howitt, Beatrice: J. Bact., xxix (1935), pp. 399-406.

[2]Beard, J. W., Finkelstein, H., Sealy, W. C., and Wyckoff, R. W. G.: Sci., lxxxvii (1938), p. 490.

[3]Shahan, M. S., Giltner, L. T., and Osteen, O. L.: Cornell Vet., xxx (1940), pp. 151-160.

the time required being two to seven days, depending upon the method of handling, guinea pigs were given subcutaneous doses of 0.1 cc. to 1 cc. in single doses or in two doses seven days apart. Ten to 30 days after the last injection of vaccine, the guinea pigs were exposed by the intracerebral injection of 0.1 cc. of 1 in 500 or 1 in 1,000 saline dilution of guinea pig-brain virus of known western type. Periodically, cross-immunity tests of virus, four in all, two from each series, were conducted in guinea pigs. Throughout, the virus in both series remained specifically western, as judged by the cross-immunity tests, and no essential difference in potency of vaccines was found at any stage, including the end, of either series. The virus in the terminal 35th passage in embryos in the "I" series remained lethal for the horse when injected intracerebrally. The infectivity of the "C" series virus for the horse was proved by intracerebral inoculations of the 59th, 124th and 226th embryo passage, further demonstrating that continuous passage in embryos had not significantly altered the infective properties of the virus.

Although equine encephalomyelitis virus may remain active in large pieces of horse brain preserved in buffered glycerin solution in the refrigerator for as long as a year, the infective titer of horse brain for guinea pigs is on the average very low and irregular. Guinea pig-brain virus may be similarly preserved for several months but during this time a gradual drop in the infective titer takes place. With continued serial passage at short intervals in guinea pigs relatively high infective titers, but some irregularities, develop. For testing vaccines, a uniformly virulent exposure virus would be an ideal attainment. As a possible aid in this direction, the following observations have been made:

In trials with 15 strains of virus guinea pig brain, frozen and dried under vacuum, a commercially made lyophile apparatus being used, was found to be active when tested within a month, but all preparations were inactive when tested after four or more months, the containers having been sealed with clamps and rubber stoppers. With the same apparatus, bacterial cultures have been preserved for several months.

Virus-bearing guinea pig brain was placed in 1 to 3 Gm. pieces, or finely ground, in Petri dishes over a quantity of P_2O_5 in a vacuum jar from which the air was withdrawn with a rotary pump. Some such preparations were not thoroughly desiccated even after a week, but after the partially dried material was sifted through a fine sieve and the material replaced in the jar, complete dryness, as determined by weighing, was finally accomplished after an average total time of ten days under vacuum. The finely divided but somewhat gummy lipoidal residue was tubed in small, soft glass tubes drawn out to tapered tips. The tip was slipped into rubber tubing connected with the pump and the evacuated tube sealed in a gas flame. The sealed tubes were stored in the refrigerator and at intervals a sample tube was opened, the contents weighed, and dilutions made in chilled physiological salt solution for prompt injection into test animals. With the dosages above described for guinea pigs and mice, it was found that some samples of rapidly and completely dried brain tissue under complete vacuum retained their infective titer for slightly more than a year, disregarding the slight immediate drop which occurred during processing. Even in tubes containing some air, the virus remained active but in diminished titer for the same time.

The somewhat irregular and relatively low virus content of guinea pig brain being considered, tests were made of embryos handled in the same manner as the guinea pig brain. Complete dryness of embryo tissue, with a resultant fluffy powder, was accomplished within five days. Here again, a slight initial drop in infective titer occurred during the drying process, but six strains of virus, including both eastern and western types, have retained an essentially uniform infective level for six to twelve months, during which time the tubes were stored in the refrigerator. Viruses stored for more than one year have not yet been tested. Two tests, in which such virus was used in dilutions comparable in virus content with the guinea pig-brain suspensions

generally used for test exposure of vaccinated guinea pigs, have been satisfactory. The probable reason for loss of virulence in tubes from which vacuum has been lost is oxidation. One strain of virus was capable of producing the disease in intracerebrally injected animals even after two months at 37.5° in the dark. Here again, failure to preserve vacuum resulted in a drop in infective titer, still without total loss of infectivity after three to six months.

The virus in both guinea pig brain and embryo tissue has been successfully maintained for months when harvested before autolytic changes had time to develop to a marked degree and the tissues stored in the freezing compartment of an electric refrigerator running at "average" temperature. Such a method of freezing is, however, relatively slow and probably some loss of virus results. Rapid freezing probably causes less marked alteration. A large vacuum-insulated chest, built on the principles of the small thermos bottle, containing alcohol and dry ice, has been found to provide convenient and efficient equipment for the rapid freezing and storing of equine encephalomyelitis virus. Fifteen strains, both eastern and western, in the form of guinea pig brain, have been frozen in this manner and titrated by means of intracerebral inoculation of mice after five to seven months of storage. Titrations of the brain tissue placed in the freezing chest were not carried out prior to freezing, but the titrations made at the intervals stated indicate that this, as well as drying over P_2O_5, may be an effective means of maintaining a stock virus for exposure or storage purposes. Either method appears to be superior to preservation in buffered glycerin solution, other things being equal.

Still another technic which may prove to be practical for preservation of the virus is the storage of the infected embryo in the shell. Some eggs so held for months in the refrigerator with no provision for prevention of evaporation of the contents of the shell have been found active.

SUMMARY

Equine encephalomyelitis virus carried through 226 serial passages in chick embryos and confined strictly to this medium for propagation remained antigenically and pathogenically indistinguishable from the same strain of virus also transmitted through embryos but periodically interrupted by passage through the horse.

Desiccation by vacuum of rapidly frozen virus, rapid freezing alone, and desiccation in vacuum by means of P_2O_5 have been tested as substitutes for buffered glycerin solution in preserving virus. Desiccation by P_2O_5 appears to be the most permanent and otherwise effective means of preservation tried.

Rapid, complete drying *in vacuo* over P_2O_5 and storage under a high degree of vacuum in the refrigerator provided a uniformly infective virus for at least one year, the maximum period of storage tested.

Studies on Equine Encephalomyelitis*

F. M. BIRCH, A.B., D.V.M.

Ames, Iowa

EIGHT STRAINS of equine encephalomyelitis virus were isolated from horses in Iowa during the summer of 1939.[1] They were recovered during the period from August 17 to September 22. The present studies were instituted to ascertain primarily what effect, if any, the repeated passage through susceptible laboratory animals might have upon the behavior of these virus strains.

For future reference these eight Iowa strains will bear the numbers 6, 11, 18, 22, 28, 30, 31 and 32.

At the time this work was begun in December 1939, the eight viruses had been well established in guinea pigs. Beck and Wyckoff[2] have carried out extensive investigations with various strains of western virus. They demonstrated that western immune guinea pigs were protected against inoculations of lethal doses of a recently acquired field strain, two strains which had been maintained by serial passages through guinea pigs and mice for a period of five years, and still another which had been subjected to 60 serial chick-embryo passages. Two of these strains originated from widely separated parts of the United States, Texas and Iowa.

ANIMAL PASSAGES

The procedure of transferring the virus was as follows: The experimentally infected guinea pigs were autopsied following death or destruction when moribund or in a state of paralysis, the brains removed aseptically and placed in sterile 50 per cent neutral glycerin and distilled water and kept refrigerated at 4° C. One-half Gm. of brain from the previous passage was ground in a sterile mortar and 50 cc. of sterile physiological saline added. The hair over the frontal bone was clipped from each of the guinea pigs and the area cleansed with alcohol. A small incision was then made through the skin over this bone after the guinea pig had been anesthetized with ether. A small opening was made in the bone with a hand drill, exposing the dura mater. Through this opening 0.1 cc. of the prepared sterile brain suspension was inoculated directly into the brain. A small strip of sterile cotton was placed over the wound and collodion applied. The guinea pigs were then placed in pens and observed daily for symptoms of encephalomyelitis.

This procedure was repeated every two weeks, inoculating each of the viruses into three susceptible guinea pigs. From time to time, bacteriologic examinations were made of the dead or destroyed guinea pigs. All bacteriologic cultures of the brain, both on serum agar and dextrose-brain broth, were negative. The autopsies showed no gross lesions in the viscera. All of the virus strains were passed once through 3 N Berkefeld filters and each strain was recovered from the filtrate.

Three or four days following inoculation, the guinea pigs were either bright and paralyzed or depressed and moribund. At these stages, salivation was often noticed. Variations from these syndromes were not common, since in all the passages throughout the year only two guinea pigs showed strikingly different symptoms, both of them dying in a state of extreme tetany.

Six of the viruses killed the susceptible guinea pigs consistently in four or five days throughout the year. The other two viruses showed irregularities in the duration of the disease they caused. Sometimes virus 6 destroyed the susceptible guinea pigs in four days, whereas, during the next passage, some of the animals lived for six or

*From the Veterinary Research Institute, Iowa State College. This report is an abridgment of a thesis submitted to the graduate faculty of Iowa State College in partial fulfilment of the requirements for the degree of Master of Science.

[1]Biester, H. E., and Schwarte, L. H.: Studies on equine encephalomyelitis in Iowa during 1939. J.A.V.M.A., xcvi (1940), pp. 719-723.

[2]Beck, C. E., and Wyckoff, R. W. G.: The antigenic stability of western equine encephalomyelitis virus. Sci., lxxxviii (1938), p. 264.

seven days before dying. This difference in the duration of the disease was noticed throughout the year and was unpredictable. Another variation manifested by the use of this virus was the rapid onset of the disease it caused and the comparatively short time in which it caused death of the host. Some of the inoculated animals died in only twelve hours following the onset of symptoms. General and systemic symptoms were usually seen at least for 48 hours before death when the other viruses were used. Virus 11, one of the strongest viruses immediately after recovery from the horse, was the only one to lose in virulence throughout the year. Twice during the year it failed to infect guinea pigs, and when this occurred, composite brain suspensions were prepared and inoculated into susceptible animals. In both cases the virus was recovered.

Figures 1 and 2 show the average duration of the disease in days for each of the virus passages. The first passage represents the duration of the disease in guinea pigs inoculated with the original horse brain; each succeeding passage was completed by using brain suspensions from the passage immediately preceding it. In the routine passage of all the viruses, a negligible number of guinea pigs died immediately or within 48 hours after inoculation without showing symptoms of encephalomyelitis. These animals were not used in computing the killing time of the viruses, nor were their brains kept for inoculation purposes.

A study of figures 1 and 2 shows a marked tendency to increased pathogenicity in seven of the eight strains of the equine encephalomyelitis viruses when passed repeatedly through guinea pigs. This increase was the most marked for about the first ten passages. Further passages produced no marked changes in virulence, and the viruses appeared to become fixed. Virus 11 appeared more virulent during some of the early passages than it did after a number of later ones.

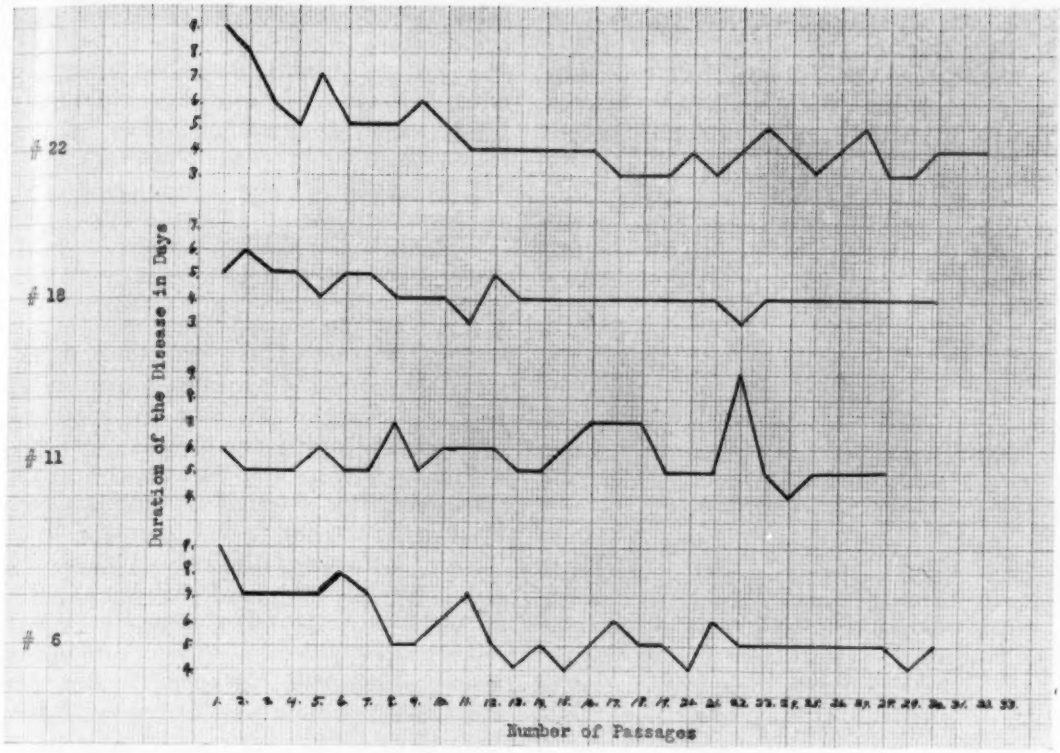
Virus 6 was of low-grade virulence during the earlier passages. Inoculations with composite suspensions made from several guinea pigs from previous passages were found necessary on several occasions to maintain this virus. The onset of the disease produced by virus 6 was sudden and of short duration, unlike the clinical courses produced by the other viruses. Although these eight strains are classified as western type viruses, strain differences in pathogenicity were manifest. Viruses 6 and 11, the two strains that behaved somewhat differently from the others, were the first two isolated during the summer of 1939.

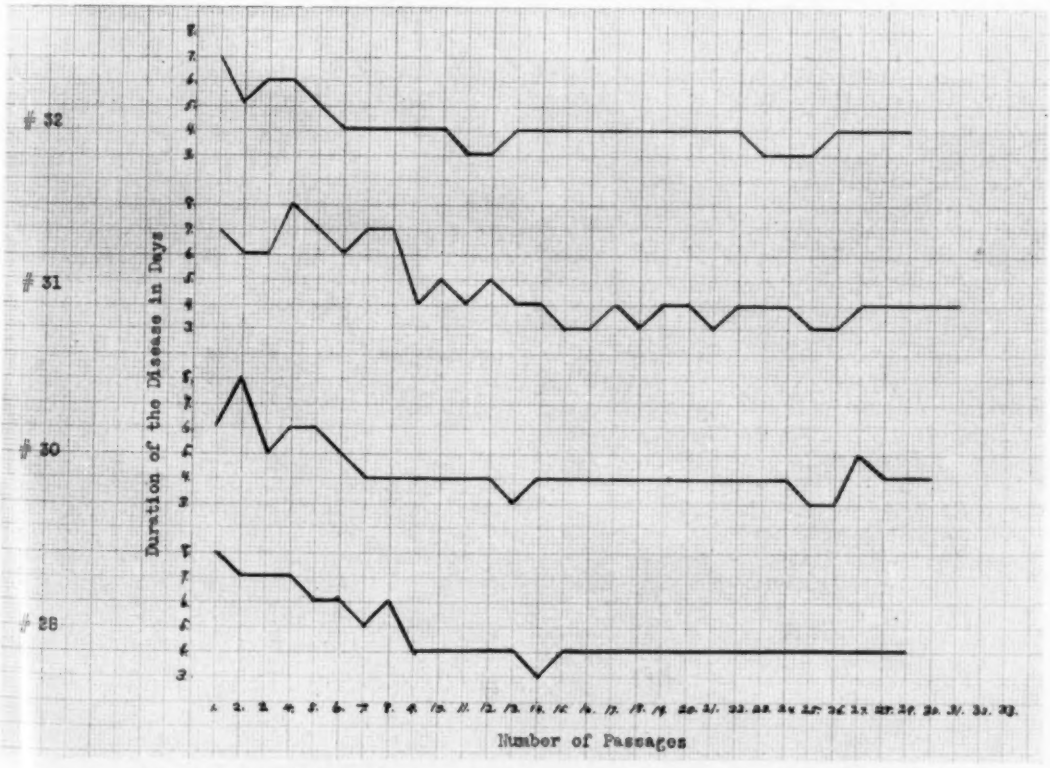
TYPING TESTS

In this project, the eight virus strains were typed after a year's serial passage through guinea pigs. The effect of the guinea pig passages upon the antigenic stability of the virus strains was also sought. The animals used for the typing tests were immunized, as recommended by Schoening,[3] as follows: Susceptible guinea pigs were given subcutaneous inoculations of 0.5 cc. of western chick-embryo vaccine in the right axillary space. Seven days later the same animals were given a similar injection in the left axillary space. Ten days after the second injection, all of these animals and three controls were given an intracerebral injection of 0.1 cc. of 1:500 brain suspension of a known western virus. The same technic was used to immunize another group of susceptible guinea pigs, the eastern vaccine and virus being used. One month following the intracerebral test dose was allowed to elapse before typing tests were begun.

In the first typing test, four controls and four eastern type-immune animals were inoculated intracerebrally with eastern virus, and four controls and four western type-immune animals were inoculated with the western virus. Each of the provisionally typed western viruses was inoculated into four controls, four eastern immune and four western immune animals (table I). Atypical results were obtained on this trial for viruses 6, 11, 18 and 28. A second typing test (table II) was then conducted in which each of these four viruses was inoculated intracerebrally into three con-

[3]Schoening, H. W.: Personal communication.





Figs. 1 and 2. Serial guinea pig passages of encephalomyelitis virus strains.

trols, six eastern immune and six western immune animals.

The eastern virus used in this work was a strain isolated in Maryland.* It was passed twice through guinea pigs before being used to test for immunity and once again before being used in the typing test. The western virus used was strain 22, isolated and typed as a western strain by Biester and Schwarte[1] in 1939. The infected horse brain from which this virus was taken came from a clinical case in southwestern Iowa.

TABLE I—The First Typing Test

Virus Strain	Eastern Immunes	Western Immunes	Controls
Known western virus (virus 22)		1—	5*
		2—	6*
		3—	7*
		4—	8*
Known eastern virus	9—		13†
	10—		14*
	11—		15*
	12—		16*
6	17*	21—	25*
	18—	22—	26*
	19—	23—	27*
	20—	24—	28*
11	29*	33—	37*
	30*	34—	38*
	31*	35—	39*
	32—	36—	40*
18	41*	45—	49*
	42—	46—	50*
	43—	47—	51*
	44—	48—	52*
22	53*		
	54*		
	55*		
	56—		
28	57*	61—	65*
	58*	62—	66*
	59—	63—	67*
	60—	64—	68*
30	69*	73*	77*
	70*	74—	78*
	71*	75—	79*
	72*	76—	80*
31	81†	85—	89*
	82*	86—	90*
	83*	87—	91*
	84*	88—	92*
32	93*	97—	101*
	94*	98—	102*
	95*	99—	103*
	96*	100—	104*

* Died following clinical manifestations.
† Died prematurely.
— =No response.

RESULTS OF THE TYPING TESTS

None of the guinea pigs used in the typing test showed any symptoms following the two subcutaneous injections of vaccine. Five of 80 animals inoculated intracerebrally with the eastern virus died within 24 hours. The controls died on the third day after showing typical symptoms. During the ten-day period following inoculation, ten more of the eastern vaccinates died. Autopsies revealed that some died of pneumonia. Brains from the others were harvested for future studies. A brain suspension was made from one of the latter and inoculated into susceptible guinea pigs.

TABLE II—The Second Typing Test

Virus Strain	Eastern Immunes	Western Immunes	Controls
6	105*	111*	117*
	106*	112—	118*
	107*	113—	119*
	108*	114—	
	109—	115—	
	110—	116—	
11	120*	126†	132*
	121*	127—	133*
	122*	128—	134*
	123*	129—	
	124—	130—	
	125—	131—	
18	135*	141*	147*
	136*	142—	148*
	137*	143—	149*
	138*	144—	
	139—	145—	
	140—	146—	
28	150*	156*	162*
	151*	157—	163*
	152*	158—	164*
	153*	159—	
	154*	160—	
	155—	161—	

* Died following clinical manifestations.
† Died prematurely.
— =No response.

*Supplied through the courtesy of H. W. Schoening, Pathological Division, U. S. Bureau of Animal Industry, Washington, D. C.

all of which died of encephalomyelitis three days later.

None of the western vaccinates died of shock after intracerebral inoculation of the virus to test for immunity. However, twelve of them died in the ten-day period following inoculation. Some of these had lesions of pneumonia. The brains of those showing symptoms of encephalomyelitis were removed and kept in glycerin for future use. The controls died five days following inoculation with the western virus.

The results of the typing tests are tabulated in tables I and II. The 44 control animals died within five days of the inoculation, and the average duration of the disease in them was 4½ days. Thirty-nine of the 56 eastern immunes died within ten days of their inoculation. One of these 39 succumbed to the shock of the inoculation. The average duration of the disease in the remaining 38 was 6½ days. Seventeen, or 30 per cent of the eastern immune guinea pigs survived for two months following inoculation and showed no symptoms of the disease at any time. Four of the 56 western immunes died in the ten-day period following inoculation, one of them of surgical shock. One western immune died 18 days following inoculation. The average duration of the disease in the four western immune animals was ten days. Fifty-one, or 93 per cent of the western immunes survived the inoculations and were free of encephalomyelitis two months later.

Effects of Ether on the Virus

Unless brains from suspected field cases of encephalomyelitis are promptly removed from the body and brought to a laboratory under suitable conditions, bacterial contamination will take place. When such contaminated brain tissue is injected intracranially into laboratory animals, death usually occurs before the virus becomes established. The attenuation or destruction of bacterial contaminants without injury to the virus content of the field sample would be an important contribution to diagnostic procedures. Filtration of brain suspension always results in a dilution of the virus and in a retention of some of it, often producing a filtrate which contains insufficient virus to infect laboratory animals. Bacterially contaminated brains that kill laboratory animals within twelve hours following intracranial inoculation often fail to kill after storage in 50 per cent glycerin. The glycerin exerts a deleterious action on the bacteria, but unfortunately, the virus already has been affected adversely during the process of bacterial invasion. A similar problem occurs in the diagnosis of rabies when contaminated tissue reaches the laboratory. Sulkin and Nagel[4] were able to overcome this obstacle in connection with rabies by treating the contaminated tissue with ether before inoculation into laboratory animals. They demonstrated the rabies virus in grossly contaminated brains by extraction with 10 per cent ether for six hours, which they found in a series of experiments to be the minimum ether concentration and time essential to control bacterial contaminants.

In an attempt to recover the equine encephalomyelitis virus from clinically infected horses, Cox, Philip, Marsh and Kilpatrick[5] centrifuged nasal washings with 10 per cent ether and stored them overnight in a refrigerator. The contaminating bacteria were effectively controlled by this procedure, but no virus could be demonstrated.

The method followed in this experiment is essentially the same as that used by Sulkin and Nagle[4] in isolating the rabies virus. A 1:100 brain suspension from a typical case of encephalomyelitis in a guinea pig produced by virus 22 was prepared and centrifuged. To each of three tubes of the supernatant fluid sufficient ether was added to make final concentrations of 10, 20 and 30 per cent ether. A fourth tube to which no ether had been added was kept as a control. All four tubes were shaken thoroughly and refrigerated for six hours. Material from each of these tubes was inoc-

[4]Sulkin, S. E., and Nagel, N.: Demonstration of rabies virus in grossly decomposed animal brains. J. Lab. & Clin. Med., xxv (1939), pp. 94-97.

[5]Cox, H. R., Philip, C. B. Marsh, H., and Kilpatrick, J. W.: Observations incident to an outbreak of equine encephalomyelitis in the Bitterroot Valley of western Montana. J.A.V.M.A., xciii (1938), pp. 225-232.

ulated intracerebrally into susceptible guinea pigs.

The controls showed typical symptoms of encephalomyelitis and died within five days of the inoculation.

With 10 per cent ether the guinea pigs recovered from the inoculations and at no time showed any symptoms of encephalomyelitis.

With 20 per cent ether two of the guinea pigs died within 24 hours of the inoculation. The third recovered from the inoculation and showed no symptoms of encephalomyelitis.

With 30 per cent ether, one of the guinea pigs died within 24 hours following inoculation. The other two showed atypical symptoms of encephalomyelitis and died in five days. The brain of one of the latter was harvested and a suspension made which was inoculated into three more susceptible guinea pigs. The latter all lived and showed no symptoms.

CONCLUSIONS

1) Seven of the virus strains increased in virulence following repeated guinea pig passage. This tendency to gain in virulence was not shown by virus 11. The clinical course produced by virus 6 differed from that caused by the other viruses.

2) All eight of the viruses were typed and shown to be strains of the western equine encephalomyelitis virus.

3) Ether added to virus-brain suspensions was viricidal. The previously described method for successful isolation of the rabies virus does not appear to be applicable to the western equine encephalomyelitis virus.

The Determination and Clinical Correlation of Variations in the Calcium, Inorganic Phosphorus, and Serum Proteins of Horse Blood*

A. HENRY CRAIGE, JR., V.M.D., and JOHN D. GADD, V.M.D.

Philadelphia, Pa., and Towson, Md.

THERE SEEMS to have been little attempt in the past to make extensive use of blood examinations on horses suspected of calcium and phosphorus disturbances. The possibility of metabolic deficiencies as a result of inadequate rations is well known to trainers and breeders everywhere, and the importance of a rich limestone soil is part of the Thoroughbred tradition. The intimate relationship between blood and tissues suggests a natural approach to diagnosis of tissue disturbances by blood examination, yet efforts in this direction apparently have met little success. From a study of the literature and the writers' experience, the fault seems to be due in some measure to the difficulty encountered in interpreting blood analyses.

REVIEW OF LITERATURE

When blood examination is resorted to in these cases, it usually is confined to serum calcium (total Ca), sometimes augmented by phosphorus determination. According to the data reported in the literature,[1-12] serum calcium may vary in normal horses from the extremes of 9 to 15 mg. per 100 cc., usually averaging between 11 and 13 mg.; and inorganic phosphorus ranges from 2 to 5 mg. per 100 cc. in mature animals, with averages between 3 and 4 mg. Higher figures are reported for phosphorus in young animals, the average for different age groups rising with decrease in age. Kintner and Holt, studying osteomalacia caused by mineral deficiency in the ration, found in cases of obvious decalcification, serum calcium figures averaging only 1 mg. per 100 cc. lower (10.2) than the average for normal animals (11.2). The range of results from the osteomalacic horses overlapped the normal range so completely that only a few of the former could be classed as definitely outside this limit. Many of the severe cases were well within the normal range, actually showing calcium levels the same as normal horses. Their results with inorganic phosphorus were analogous, the osteomalacic horses revealing, in general, levels slightly higher than those of normal animals, but not sufficiently increased, with the exception of a few cases, to be considered definitely abnormal.[6] V. B. Jones,[12] in reporting blood examinations for serum calcium and inorganic phosphorus in a varied group of equine clinical subjects, concluded, "The figures appeared so varied that it does not seem possible to differentiate the normal from the abnormal." Clearly the difficulty lies in identifying possible alterations of slight degree in the face of comparatively wide ranges of normal variation.

Physiologic Variation in Blood Calcium and Phosphorus.—Environmental and physiologic influences account for much of the diversity in blood findings. Foremost in the environmental factors is the daily intake of calcium and phosphorus in the feed. Also, the supply of vitamin D should be mentioned, as well as

*From the School of Veterinary Medicine, University of Pennsylvania, and the Towson Veterinary Hospital (Towson, Md.).

[1]Abderhalden, E.: Zur quantitativen vergleichenden Analyse des Blutes. Zeit. Physiol. Chem., xxv (May 1898), p. 65.

[2]Amadon, R. S.: Blood Analysis. Vet. Ext. Quar. Bul. 56, Univ. of Pennsylvania (Oct. 1934).

[3]Carlström, B.: Über die Ätiologie und Pathogenese der Kreuzlähme des Pferdes. Skand. Arch. Physiol., lxii (1931), p. 1.

[4]Dimock, W. W., and Healy, D. J.: The chemical character of the blood and urine of colts. J.A.V.M.A., lxxxiii (Dec. 1933), p. 806.

[5]Errington, B. J.: Variations in inorganic phosphorus and calcium content of the blood of horses. Cornell Vet., xxvii (Jan. 1937), p. 1.

[6]Kintner, J. H., and Holt, R. L.: Equine osteomalacia. Philippine J. Sci., xlix (Sept. 1932), p. 1.

[7]Pearson, P. B.: Inorganic phosphorus of horse serum. The effect of age and nutrition. J. Biol. Chem., cvi (Aug. 1934), p. 1.

[8]Pearson, P. B., and Catchpole, H. R.: Correlated studies of the partition of calcium and inorganic phosphorus in the blood sera of Equidae. Amer. J. Physiol., cxv (March 1936), p. 90.

[9]Robinson, W. G.: The mineral composition of the blood serum of the horse and pig. Univ. of Cambridge Inst. Ani. Path., 3rd rpt. of director (1932-33), p. 156; abstracted from Chem. Absts.

[10]Blunn, C. T., Howell, C. E., and Caldwell, R. W.: The calcium inorganic phosphorus and magnesium content of the blood serum of young horses. J. Nutri., xx (July 1940), p. 1.

[11]Stewart, J., and Holman, H. H.: The blood picture of the horse. Vet. Rec., lii (March 2, 1940), p. 157.

[12]Jones, V. B.: Navicular disease. Vet. J., xciv (Aug. 1938), p. 347.

the extent of exposure to sunlight. As to physiologic sources of variation in blood calcium and phosphorus, we have mentioned age of the animal. Others will be developed in the course of this presentation.

Under natural conditions variations in the intake of calcium and phosphorus are the result of variations in the soils upon which the feeds have been grown. Soil deficiency functions to limit the plant content of the deficient element. A recent state-wide survey of soils in Maryland indicates that phosphorus deficiency is widespread, many areas being doubly handicapped by low calcium as well.[13] Nor is this situation confined to one state. The U. S. Department of Agriculture has called attention to general soil deficiency in these elements, blaming uncontrolled erosion and traditional methods of cropping for all but exhausting vast acres of farmland.[14, 15]

Another common source of deficiency is the use of feeds naturally low in these elements. Timothy hay, a time-honored favorite among horsemen, is notably poor in calcium, as compared with the legumes. Oats and corn, frequently used as supplements, are not much richer in phosphorus than average timothy hay, and they contain very little calcium. It is evident that a ration composed entirely of timothy hay, corn, and oats, together with mediocre pasturage, runs the danger of approaching deficiency levels of calcium and phosphorus.[16]

In addition to richness of the soil and variety of crops, it is necessary to consider other factors contributing to determine the adequacy of the feed. Weather conditions may be favorable or unfavorable to transfer of these elements from the soil to the plant. Then, there is the growth stage at which the plant is cut or used for grazing, and care in preparing the hay, to mention some of the sources of variation in mineral content. Apart from the mineral content, vitamin D supply is an important item. In a recent review of vitamins in the nutrition of farm animals, J. H. Jones (1940) pointed out that deficiency in D is more than a theoretical possibility,[17] and in view of the rôle of this vitamin in the metabolism of calcium and phosphorus, its supply must be considered in conjunction with the supply of the elements.* Exposure to sunlight, season of the year, pigmentation of the skin and thickness of the coat all have a bearing on the quantity of vitamin D in the body.[18] No attempt is made at this time to explore the question of dietary requirements for the horse. The reader is referred to a recent paper by Schmidt[19] for a comprehensive review of the literature concerning calcium and phosphorus deficiency in horses and cattle.

Variations in age affect the blood phosphorus level and also the extent of blood reaction to alterations in the ration. Young, growing animals normally show higher blood phosphorus than mature horses. According to Pearson,[7] the phosphorus in foals less than 1 year old runs about 2 mg. per 100 cc. higher than in mature horses. In his study, findings on yearlings and 2-year-olds were about 1.0 and 0.5 mg. per 100 cc., respectively, in excess of those for older animals. A similar trend was reported by Blunn, Howell, and Caldwell,[10] who noted an initial rise in phosphorus from the level at foaling to a maximum at 2 to 4 weeks old. Furthermore, blood calcium and phosphorus are more susceptible to dietary variation in young horses than in mature,[5] although mature animals which have been fed deficient rations for a long period respond to increased intake of the deficient element.[6] Phosphorus reductions were attributed by Pearson to dietary deficiency where mature horses were grazed on parched pastures over a long period. Evidently age is the important determinant in susceptibility to deficiencies in the ration, but horses of all ages suffer from inadequate supply of calcium and phosphorus.

Pearson and Catchpole[8] found breed to be a factor in determining the phosphorus, but not the calcium. They reported averages for Shetland ponies of 3.96 mg. per 100 cc. of serum phosphorus, as compared to the larger breeds, with averages of 3.29 (Percherons) and 3.30 (Arabs).

Findings in Disease.—Although many papers have appeared in the past on enzoötic bone disease among horses, few writers have paid more than perfunctory attention to the effects

[13]Thomas, R. P.: Some results of rapid soil testing in Maryland. Report of the Maryland Crop Improvement Association (Baltimore, January 11, 1939), p. 16.

[14]Truog, E.: Soil acidity and liming. U. S. Dept. of Agriculture Yearbook, "Soils and Men," House Doc. 398 (Washington, 1938), pp. 563-580.

[15]Pierre, W. H.: Phosphorus deficiency and soil fertility. *Ibid.*, pp. 377-396.

[16]Morrison, F. B.: Feeds and Feeding. (20th ed., Ithaca, N. Y., Morrison Publishing Co., 1939), pp. 105, 205.

[17]Jones, J. H.: The relation of vitamins to the nutrition of farm animals. J.A.V.M.A., xcvi (March 1940), p. 327.

*Other components of the feed have been scrutinized from the standpoint of their deficiency causing lameness. Particular attention has been paid vitamin A in this respect. A report which appeared since this writing (Howell, C. E., Hart, G. H., and Ittner, N. R.: Vitamin A Deficiency in Horses, Amer. J. Vet. Res., ii (Jan. 1941), pp. 60-74), provides challenging evidence in support of A avitaminosis as a potent source of articular disease among horses.

[18]Hess, A. F.: Newer aspects of the rickets problem. J.A.M.A., lxxviii (April 22, 1922), p. 1177.

[19]Schmidt, H.: Calcium and phosphorus deficiencies in cattle and horses: clinical picture, treatment, and prevention. J.A.V.M.A. xcvi (April 1940), p. 441.

of pathologic processes on blood calcium and phosphorus levels. The work of Kintner and Holt, previously mentioned, constitutes a notable exception. In contrast to the usual findings, Berge and Pallaske[20] found in a case of osteodystrophia fibrosa high serum calcium (17.8-18.8) and normal phosphorus (2.18-3.52 mg. per 100 cc.). Errington[5] found diminutions in blood phosphorus accompanying bone disease, but the calcium remained unchanged. Liebengood[21] described two cases of occult lameness which responded to bone meal in the feed; his analyses made before treatment showed calcium to be 10.9 and 9.59 mg. per 100 cc., respectively, while phosphorus was 2.38 and 3.12, in the same order.

Published data indicate that azoturia is frequently associated with moderate hyperphosphatemia (averaging over 6 mg. per 100 cc. of phosphorus, in mature horses),[22] although both phosphorus and calcium are more often unaffected.[3] Stewart, Gordon, and McCallum[23] observed rises in blood phosphorus, and concomitant reduction in calcium, with the approach of death due to "grass sickness."

Lameness.—There is an opinion coming to the fore that the common lamenesses of the horse, such as navicular disease, spavin, ringbone, etc., should be regarded as manifestations of a generalized bone weakness, recognized in more advanced stages as rickets, osteomalacia and osteoporosis. Theiler[24] has classified the various bone diseases of domestic animals, calling attention to the progressive nature of the changes, which begin insidiously as a gradual decalcification, and terminate with the extreme bone changes of osteomalacia and osteoporosis. Many cases never reach the later stages, revealing the earlier changes in the lesions causing lameness. After considering the "various crippling and disabling conditions" among horses, Mitchell[25] came to the conclusion that they are all "local manifestations of one general disease."

As to the cause of these bone changes, several theories have been advanced from time to time. They have been reviewed by Campbell[26] in historical sequence down to the current explanations, which lean heavily on nutritional defects. Mitchell favors the idea of nerve degeneration and local circulatory disturbances as the fundamental changes, but he regards faulty rations as a contributory cause of the disease. In connection with this, Dimock and Errington[27] recently reported osteochondral deformities associated with nerve degeneration in "wobblers," victims of a disease affecting young horses, characterized by marked incoördination of gait. Autopsy revealed in many of their cases that the nerve changes were secondary to deformity of the spinal column. These writers alluded to nutritional defects as a possible underlying cause.

Kelser and Callender[28] described a form of arthritis, common in the army horses and mules in Panama, in which degenerative changes involve the articular cartilages without necessarily affecting other parts. They suspected nutritional deficiency, possibly mineral imbalance, as the cause of the disease. Much of this research was under the direction of Col. C. W. Greenlee, who has been studying this problem for many years. In a recent review,[29] he expressed the conclusion, "Prevention of skeletal diseases of horses is dependent largely upon the following: proper fertilization of the soil upon which feedstuffs are grown; harvesting at the proper time; proper curing and storing; feeding an adequate ration." The whole literature on equine osteomalacia (or osteodystrophia) seems to imply that the usual cause of frank demineralization is dietary deficiency in either calcium or phosphorus. If it is true, as the evidence suggests, that the same disturbance operates in common lameness and advanced bone disease, then there is reason to suspect the same cause.

It is logical to expect some alteration in the calcium and phosphorus of the blood in cases with bone lesions which are the result of dietary deficiency in these elements. If the ration has been so poor in them as to deny normal growth to the bones, the blood should reflect this adequacy. Since these cases are usually chronic, the result of a prolonged period of insufficiency, the chances are that the blood deficits are not large. This is verified by the findings of Kintner and Holt, mentioned in the second paragraph of this paper.

[20] Berge and Pallaske: Osteodystrophia Generlisata beim Pferd. Berl. Tierärztl. Wochenschr., 1 (June 8, 1934), p. 385.

[21] Liebengood, H.: Phosphorus deficiency lameness in horses. Vet. Med., xxxiv (Aug. 1939), p. 490.

[22] Grzycki, S.: Ueber das Wesen und die Therapie der Haemoglobinuria paralytica beim Pferde. Berl. Tierärztl. Wochenschr., 1 (Nov. 30, 1934), p. 789.

[23] Stewart, J., Gordon, W. S., and McCallum, J. W.: Grass sickness in horses—biochemical investigation. Vet. Rec., lii (March 30, 1940), p. 237.

[24] Theiler, A.: The osteodystrophic disease of domesticated animals. Vet. J., xc (April 1934), pp. 143-175, and (May 1934), pp. 183-206.

[25] Mitchell, W. M.: The problem of "rheumatic disease" (osteoarthritic and allied conditions) in the horse. Vet. Rec., xlvii (December 14, 1935), p. 1501.

[26] Campbell, D. M.: Lameness in horses—its relation to their feed. Vet. Rec., xlix (Feb. 6, 1937), p. 139.

[27] Dimock, W. W., and Errington, B. J.: Incoördination of Equidae: wobblers. J.A.V.M.A., xcv (Sept. 1939), p. 261.

[28] Kelser, R. A., and Callender, G. R.: Equine degenerative arthritis. Vet. Med., xxxiii (July 1938), p. 307.

[29] Greenlee, C. W.: The skeletal diseases of horses and their relation to nutrition. Cornell Vet., xxix (April 1939) p. 115.

The observations of Humphrey[30] on race horses fed rations of different mineral content illustrate that serious deficiencies do exist among horses on our well-equipped farms, and also that feeding rations rich in calcium and phosphorus pays profits in better racing and fewer casualties. The chief problem confronting the diagnostician is determining whether or not a horse shows deficiency in these elements.

Biochemic Influences.—The modern concept that calcium exists in the blood in more than one form dates from the work of Rona and Takahasi (1913). By dialysing blood serum, they distinguished between diffusible and nondiffusible fractions, attributing the latter to a combination of calcium with the serum proteins.[31] Much study has been devoted to the nature and extent of this relationship. Improved methods for the separation of the two forms have been introduced by Greenberg and Gunther[32] (ultrafiltration) and Greene and Powers[33] (vivadialysis). With the addition of a biologic method for determining directly the concentration of an ionized fraction, McLean and Hastings[34] made an important contribution toward understanding the significance of variations in blood calcium.

It is agreed rather generally now that calcium is present in the blood in a variable proportion of a fraction physiologically inert and an active fraction, calcium-ion. It is alterations in the latter which reflect physiologic disturbances in the supply and regulation of this element in the body. Calcium in the inactive portion is regarded as bound in a form not directly available to the tissues. As Rona and Takahashi surmised, a protein combination accounts for the principal part of the inactive nondiffusible fraction, while a small residue appears as a nonionic, diffusible fraction, presumably calcium citrate.

In a given case the proportion of blood calcium in these various fractions depends upon several interrelated factors. Ionic calcium (Ca^{++}) is in equilibrium with calcium proteinate and with free protein. This equilibrium also embraces calcium citrate (nonionic diffusible), as well as the citric acid and magnesium in the blood. Furthermore, it is known that variations in the temperature and pH of the blood influence this equilibrium, although for practical purposes these factors may be disregarded, as constant under the conditions met in this study.[34]

From the reported levels of magnesium and ctiric acid in horse blood, it may be concluded that the combined influence of these factors on the ionization of calcium is negligible. Serum magnesium ranges from 1.0 to 2.5 mg. per 100 cc.[6, 10, 11] The single reference to citric acid indicates a range from 4.8 to 5.7 mg. per 100 cc., determined by Hirschlaff-Lindgren[35] on a small group. In animal species with a relatively constant blood citrate level, like man, no special consideration need be given the small calcium fraction as citrate. Some species, such as the rabbit, however, exhibit a widely variable blood citrate, at times attaining high levels.[36, 37] The total and diffusible calcium vary directly with these changes in citrate, but Ca^{++} remains unaffected.[34]

To our knowledge, it has not yet been determined whether the horse follows man or the rabbit, in constant or variable blood citric acid. Assuming that the diffusible calcium fraction consists essentially in Ca^{++} and Ca-citrate, the uniformity in the diffusible calcium of horse blood, reported by Pearson and Catchpole, leaves little room for wide variation in nonionic, diffusible calcium. While the data of Hirschlaff-Lindgren need verification, especially as to the effects of physiologic influences, if they can be accepted it is evident that the influence of magnesium and citric acid on the calcium-protein equilibrium must be slight and, being in the opposite direction, they cancel each other. The remaining factors may be correlated following the formula of McLean and Hastings for calculating Ca^{++} level in serum from total calcium and protein.

No reference was found to direct determinations of Ca^{++} in horse blood, nor were we able to find figures on serum proteins obtained simultaneously with the calcium. It is known that the proteins vary, both in total concentration and in albumin: globulin (A:G) ratio, with the age of the animal and under the influence of pregnancy. It has been suspected that seasonal change also plays a part. Disease and infection, however, exert profound effects on the serum proteins, particularly in horses.[38]

[30]Humphrey, R. L.: Some reasons why the E. B. McLean horses headed the list of money winners in 1928. Address before the 18th annual meeting of the Horse and Mule Association of America, December 1, 1937.

[31]Rona, P., and Takahashi, D.: Beitrag zur Frage nach dem Verhalten des Calciums in Serum. Biochem. Zeit., xlix (1913), p. 370.

[32]Greenberg, D. M., and Gunther, L.: On the determination of diffusible and nondiffusible serum calcium. J. Biol. Chem., lxxxv (Jan. 1930), p. 491.

[33]Greene, C. H., and Powers, M. H.: The distribution of electrolytes between serum and the *in vivo* dialysate. J. Biol. Chem., xci (April 1931), p. 183.

[34]McLean, F. C., and Hastings, A. B.: The state of calcium in the fluids of the body. 1. The conditions affecting the ionization of calcium. J. Biol. Chem., cviii (Jan. 1935), p. 285.

[35]Hirschlaff-Lindgren, B.: Die Zitronensäugehalt des Pferdeserums. Skand. Arch. Physiol., lxxvi (March 1937) p. 15.

[36]Kuyper, A. C., and Mattill, H. A.: Some aspects of citric acid metabolism. J. Biol. Chem., ciii (Nov. 1933), p. 51.

[37]Lindholm, H.: Der Citronensäuregehalt im Serum von Kaninchen unter spezieller Berücksichtigung vershiedener Diäten. Skand. Arch. Physiol., lxx (1934), p. 295.

[38]Howe, P. E.: The function of the plasma proteins. Physiol. Rev., v (Oct. 1925), p. 439.

The total proteins of horse serum, determined by various methods, are reported to range normally from about 5 to 8 Gm. per 100 cc.; the few references to A:G ratio vary from the extreme 0.6 to 1.8, presumably in normal horses.[39-43] Variations of this magnitude emphasize the impossibility of drawing conclusions regarding the Ca^{++} concentration from total calcium findings without information on the proteins.

In an earlier paper Pearson and Catchpole[44] reported observing fluctuations in the proportion of diffusible and nondiffusible calcium associated with variations in the inorganic phosphorus of the blood. Comparing groups of horses with different phosphorus levels, they found the hypophosphoremic group showing higher diffusible calcium (averaging 8.09 mg. per 100 cc.) than the horses with normal phosphorus (diffusible Ca averaging 7.15 mg. per 100 cc.). This difference was ascribed in part to reduction in the nondiffusible fraction and in part to rise in total calcium. Diffusible inorganic phosphorus also showed a higher proportion to total inorganic phosphorus in the hypophosphoremic group (94%) than in the group with a normal inorganic phosphorus (89%). In their later report[8] the same investigators found no significant correlation between total calcium and total inorganic phosphorus, and the correlation between the diffusible fractions was indefinite.

The reciprocal effect of primary alterations in either calcium or phosphorus of the blood on the other element is well established from both laboratory and clinical observation. Conditions resulting in a significant rise in phosphorus are accompanied by a fall in calcium. Conversely, when the increase affects calcium directly, phosphorus is reduced concurrently. For reviews of this literature consult the sections on calcium and phosphorus in the text by Peters and VanSlyke,[45] and the monograph by Schmidt and Greenberg.[46] Due presumably to the overshadowing effect of protein fluctuations, this relationship is more readily apparent in animals with a high blood phosphorus (*e.g.*, over 5 mg. per 100 cc.), as in young animals and cases of nephritic retention. Even so, it has been widely adopted as a basis for estimating the state of calcium and phosphorus in the body. Howland and Kramer[47] (1922) advocated the Ca x P (both as milligrams per 100 cc.) product as a constant in the diagnosis of infantile rickets. Holt, LaMer, and Chown[48] (1925) went a step further to calculate the ion product on the basis of the solubilities of the resulting salts. These methods assume all the blood calcium to be in equilibrium with the phosphorus as either tricalcium phosphate, or various proportions of monobasic, diabasic and tribasic salts. They make no allowance for the equilibrium between calcium and protein.

Salvesen and Linder[49] (1923) studied the simultaneous effects of both phosphorus and proteins on the blood calcium of human subjects with heart disease and nephritis, presumably disturbances not affecting calcium regulation. Their observations were extended by Peters and Eiserson[50] (1929) and coördinated statistically into a simple linear equation. This equation expresses, in terms of the phosphorus and protein levels in a blood specimen, the amount of calcium (total Ca) which should be present, provided there is no disturbance in calcium regulation. While admittedly open to criticism, this method of interpreting calcium and phosphorus findings has much to recommend it to the clinician. It suggested the possibility of finding a similar basis for interpreting variations in the blood of horses. Preliminary tests indicated little agreement using the Ca x P product of Howland and Kramer; coördinating the calculated Ca^{++} and inorganic phosphorus, in the manner described in this report, seemed to offer a better solution.

---

[39]Hamarsten, O.: Ueber das Paraglobulin. Arch. f. d. ges. Physiol., xvii (1878), p. 413.

[40]Howe, P. E.: The use of sodium sulphate as the globulin precipitant in the determination of proteins in the blood. J. Biol. Chem., xlix (Nov. 1921), p. 93.

[41]Lewinski, J.: Beobachtungen über den Gehalt an Serumalbumin, Serumglobulin und Fibrinogen. Arch. f. d. ges. Physiol., c (Dec. 1903).

[42]Porges, O., and Spiro, K.: Die Globulins des Blutserums. Beitr. z. Chem. Physiol. Path., iii (1903), p. 277.

[43]Wladasch, A.: Das Verhältnis von Albumin zu Globulin im Blutserum gesunder Haustiere. Biochem. z., cclxxxvii (Oct. 27, 1936), p. 337.

[44]Pearson, P. B., and Catchpole, H. R.: The partition of calcium and inorganic phosphorus in equine serum. Amer. Soc. Ani. Prod., Proc. 27th ann. meet. (1934), p. 84; abstracted from Chem. Absts.

[45]Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry (Williams and Wilkins, Baltimore, 1932, vol. 1), pp. 805 and 1090.

[46]Schmidt, C. L. A., and Greenberg, D. M.: Occurrence, transport, and regulation of calcium, magnesium and phosphorus in the animal organism. Physiol. Rev., xv (July 1935), p. 297.

[47]Howland, J., and Kramer, B.: Factors concerned in the calcification of bone. Tr. Amer. Pediat. Soc., xxxiv (1922), p. 204.

[48]Holt, L. E., Jr., LaMer, V. K., and Chown, H. B.: Studies in calcification. 1. The solubility product of secondary and tertiary calcium phosphate under various conditions. J. Biol. Chem., lxiv (July 1925), p. 509.

[49]Salvesen, H. A., and Linder, G. C.: Observations on the inorganic bases and phosphates in relation to the protein of blood and other body fluids in Brights disease and in heart failure. J. Biol. Chem., lviii (Dec. 1923), p. 617.

[50]Peters, J. P., and Eiserson, L.: The influence of protein and inorganic phosphorus on serum calcium. J. Biol. Chem., lxxxiv (Oct. 1929), p. 155.

PROCEDURES

During the past two years 330 horses have been studied in connection with this project, some of which were clinically healthy and sound of limb, some definitely pathologic. Most of the blood specimens were obtained in Maryland within a radius of 50 miles from Baltimore, very largely from Thoroughbreds. One of the collaborators (G.) made most of the physical examinations of the animals, and where pathology was encountered followed the cases in the course of his practice. Some were hospital subjects at the Veterinary Hospital, University of Pennsylvania, and a few were submitted by other interested veterinarians.

For this study jugular blood specimens were collected in pyrex centrifuge tubes of 50-cc. capacity. After filling they were stoppered with molded rubber caps and set aside until coagulation was complete. Specimens collected at a distance from the laboratory were transported by mail, which resulted in delay up to 24 hours before analyses could be started. In most cases the samples presented at the laboratory appeared to be in good condition, with a clear yellow serum separated from the shrunken coagulum and no evidence of hemolysis. In a few instances where the serum was tinged with hemoglobin, the degree of discoloration was noted and its possible influence on the inorganic phosphorus and protein content was considered.

In order to determine the effect of delay on the results obtained, specimens of blood were collected from three horses at the laboratory and kept at room temperature (about 22° C.) for several days. Aliquot parts were examined for calcium, inorganic phosphorus and total protein at various intervals after collection. Also, immediately after separation, some of the serum from these specimens was removed from contact with the clot and stored in a refrigerator (about 10° C.) for several days. Again, following the procedure for the routine handling of specimens necessitated by the conditions of this study, aliquots of serum which had stood in contact with the clot overnight were separated and placed in the refrigerator for another day before repeating the determinations.

It was found that phosphorus showed only a slight increase in the serum which had been in contact with the clot at room temperature for one day (+0.3 mg. per 100 cc.), but this figure rose rapidly as the period of standing was lengthened. Serum removed promptly from the clot, however, showed but slight phosphorus increase after four days in the refrigerator (+0.2). Calcium levels showed little change in any except the serum which remained in contact with the clot four days at room temperature, when the results were definitely lower (—0.5). Under the same conditions protein levels were affected only slightly, and fractionation determinations, therefore, were not made.

It was concluded that the only effect of a delay of 24 hours or less in beginning the chemical examination was a slight rise in the inorganic phosphorus, presumably from the hydrolysis of organic phosphorus. The magnitude of this error is not great enough to affect the results significantly, especially since it is more or less constant. It must be remembered, however, that higher temperatures and longer delay may result in very much greater increases in the inorganic phosphorus. The method recently adopted is to separate the serum from the clot before mailing the specimen to the laboratory, in cases where the delay due to transit is likely to be as long as 24 hours. Results following this rule indicate slightly lower phosphorus figures, but except where the delay is more than 24 hours, the difference is not great. In delayed shipments it is possible to obtain more reliable results on specimens of freshly separated serum than on coagulated whole blood.

Total calcium was determined by the Clark and Collip modification[51] of the method introduced by Kramer and Tisdall.[52] For inorganic phosphorus we employed the stannous chloride procedure of Youngburg and Youngburg.[53] Proteins were determined following the micro-Kjeldahl technic used in the laboratory of the Philadelphia General Hospital.[54] Serum in 1-cc. aliquots was digested, and the resulting ammonia distilled and titrated. Fractionation consisted in precipitation of the globulins from sodium sulfate solution, 22 per cent, after the method of Howe.[40] Non-protein nitrogen was determined on the first 100 cases, some by the micro-Kjeldahl, colorimetric method of Folin and Wu,[55] and some by macro-Kjeldahl digestion of a trichloracetic acid filtrate and titration. The results ranged approximately from 20 to 45 mg. per 100 cc., averaging 30, s.d. 5, in agreement with our findings on whole blood

[51]Clark, E. P., and Collip, J. B.: A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., lxiii (March 1925), p. 461.

[52]Kramer, B., and Tisdall, F. F.: A simple technique for the determination of calcium and magnesium in small amounts of serum. J. Biol. Chem., xlvii (Aug. 1921), p. 475.

[53]Youngburg, G. E., and Youngburg, M. V.: Phosphorus metabolism. 1. A system of blood phosphorus analysis. J. Lab. & Clin. Med., xvi (Nov. 1930), p. 158.

[54]Wilson, D. W., and others: Manual of Clinical Biochemistry. (Graduate School of Medicine, Univ. of Pennsylvania, 1937), p. 31.

[55]Folin, O., and Wu, H.: A system of blood analysis. J. Biol. Chem., xxxviii (May 1919), p. 81.

and those reported in the literature.[6, 11, 56, 57, 58] Further examinations were made omitting a separate non-protein nitrogen determination, making the correction by subtracting an assumed 0.3 mg. of nitrogen per cubic centimeter from total serum nitrogen. The remaining nitrogen was calculated as protein, the factor 6.3 being used for the conversion. Routine analyses were made in duplicate, with further repetition when the two findings failed to agree closely.

Results in Normal Animals

The results obtained from 410 examinations on 330 horses were examined with regard to the effects of sex, age, season of the year, feeding, pregnancy and pathology. Insofar as reliable information could be obtained on the histories of the animals examined, the analytic findings were collated from these various points of view. Unfortunately, the history was not complete in several instances, necessitating omission of the related analytic figures from the compilations. No variations could be attributed to difference in breed, but considering the small proportion of cases taken from breeds other than the Thoroughbred, our results do not afford an adequate foundation for judgment.

Sex.—No differences in chemical composition could be attributed to sex among entire and castrated males, and nonpregnant females. Mares in foal, however, and mares recently delivered of foal showed distinct variations from the preceding, as discussed in the section on pregnancy.

Age.—As other writers have indicated, the level of inorganic phosphorus in the blood varies with the age of the animal. The maximum levels, seen in the youngest foals, recede gradually as age advances and the growth rate decreases. Most authorities are of the opinion that the horse reaches physical maturity about the fifth year, in conformance with the completion of the dental arcade. From a maximum average in this series of 7.1 mg. per 100 cc. of serum inorganic phosphorus for foals less than 3 months old, the average receded with advancing age to a minimum of 3.1 mg. per 100 cc. for males and nonpregnant females over 5 years old. Further change was not found to be associated with further advance in age (table I).

The serum proteins are affected in a different manner. The only age group to show a significant variation from the average in total serum-protein level consists of the foals less than 3 months old. The ten foals in this group had protein levels ranging from 4.9 to 5.6 Gm. per 100 cc. of serum, with an average of 5.1 as compared with a range from 5.4 to 6.8, averaging 6.1, for foals between 3 and 6 months old. Groups of more advanced age did not vary significantly from the latter average, which may be compared with the general average of 6.1 for all normal animals over 3 months old, excluding pregnant mares (table I).

Apparently this protein deficit in young foals is due largely to a deficiency of serum globulin, which averaged 1.9 Gm. per 100 cc. in the youngest group. This tendency also may be suspected in the next age group (3 to 6 months old), which shows a globulin level averaging 2.4, as compared with the general average of 2.7 for all normal horses over 6 months old. Both these groups of young foals were found to possess albumin levels close to the general average of 3.4 Gm. per 100 cc. of serum, the first slightly lower (3.2), and the second as much higher (3.6). Consequently, the A:G ratio which is highest for the youngest group, averaging 1.7, was lower for the group of foals 3 to 6 months old, averaging 1.5, and reached a constant with the foals over 6 months old, averaging 1.3.

Age does not appear to be a factor in determining the serum content of total calcium. Averages for the age groups at which different levels of phosphorus were focused show no significant deviation from the general calcium average of 12.2 mg. per 100 cc.

Seasons.—The averages obtained for to-

[56] Holt, R. L., and Reynolds, F. H. K.: Blood chemistry of the horse. J.A.V.M.A., lxv (Sept. 1924), p. 732.

[57] Hayden, C. E., and Fish, P. A.: The normal blood of some domesticated animals. Cornell Vet., xviii (1928), p. 197.

[58] Skranek, G., and Otter, K.: Untersuchungen über den Reststickstoffgehalt im Blutplasma des Pferdes und des Rindes. Arch. f. wiss. u. prakt. Tierheilk., lvii (1928), p. 567.

TABLE I—Effects of Age and Pregnancy on the Composition of the Blood Serum in Normal Horses

Mean values for various age groups, ± standard deviation from the mean.

AGE GROUP	Ca (MG. %)	Ca++ (MG. %)	P (MG. %)	Fv=0*		EXAMS.	PROTEIN (GM. %)	EXAMS.	ALBUMIN (GM. %)	GLOBULIN (GM. %)	A:G	EXAMS.
				A. D.	S. D.	ANS.		ANS.				ANS.
Less than 3 mo.		6.6± .2	7.1± .4	+ .08	.17	9/9	5.1± .2	10/10	3.2± .3	1.9± .3	1.7± .4	10/10
3-6 mo.			6.6± .6	— .40	.53	13/13			3.6± .2	2.4± .4	1.5± .2	16/16
6-12 mo.			5.8± .7	— .01	.23	69/48						
1-1½ yr.			5.2± .8	— .02	.39	17/17						
1½-2 yr.	12.2± .5	6.1± .3	4.8± .5			20/20	6.1± .5	315/243	3.4± .4	2.7± .4	1.3± .3	159/157
2-3 yr.			3.8± .6	+ .06	.26	12/12						
3-4 yr.			3.5± .5	+ .13	.30	14/13						
4-5 yr.			3.3± .5	+ .12	.33	10/10						
More than 5 yr.												
Males†			3.1± .6	+ .03	.39	48/48						
Pregnant females‡		5.7± .4	3.0± .6	— .26	.53	61/39	6.7± .5	62/40	2.9± .4	3.9± .6	0.8± .2	42/26
Total number of examinations/number animals examined						283/239		387/290				227/209

*Fv = Ca++ + 0.4 P — C_{age}; a.d. (average deviation) and s.d. (standard deviation) from Fv = 0.

†Males and nonpregnant females.

‡Pregnant females, and those having foaled within the year.

All animals were diagnosed normal as to any disturbance likely to affect the results. In the case of the proteins, subjects with chronic and localized bone lesions were included; but Ca, P and Fv findings were omitted from this summary. No subjects evincing symptoms of systemic involvement were included in any of the summaries (*e.g.*, cases with fever, diarrhea, respiratory distress). Only normal animals, free from any symptoms of disease, were used for the data on Ca, Ca++, P and Fv.

tal serum protein during each month (over a two-year period) suggest a tendency toward a temporary fall in the protein level at the change of seasons, both spring and autumn. Results from 303 examinations on 230 males and nonpregnant females, in age over 3 months, are summarized in figure 1 and table II. They indicate that there is a distinct gradual decline from the high winter level of February (6.31 Gm. per 100 cc.) during the succeeding months, to the minimum of May (5.80), followed by a rapid return to a high level in June (6.38) which is maintained through September. When the cooler weather comes, there is again a reduction, less marked than in the spring, and soon followed by a rise to the higher levels of winter.

Fractional figures were obtained on some of these animals and summarized by seasons, based on the monthly fluctuations in

TABLE II—Seasonal Trends Portrayed in Figure 1

MONTH		1	2	3	4	5	6	7	8	9	10	11	12
PROTEIN	Mean	6.22	6.31	6.13	5.90	5.80	6.38	6.40	6.30	6.42	6.12	6.14	6.21
	Cases	21	33	31	26	22	9	15	12	28	23	22	36
Ca	Mean	12.12	12.55	12.15	12.04	11.61	12.56	12.51	12.02	12.16	12.08	12.52	12.38
	Cases	18	40	23	32	24	7	12	11	23	31	29	40
Ca++	Mean	5.92	6.07	6.02	6.10	5.86	6.21	6.03	5.94	5.97	5.87	6.26	6.17
	Cases	17	39	22	28	18	7	12	10	24	30	28	41
Fv*	Mean	+.12	+.06	+.14	+.16	—.15	—.03	+.24	—.15	—.11	—.16	—.06	—.00
	Cases	4	25	13	21	12	3	8	4	14	14	16	27

*Ca++ + 0.4 P — C_{age} = Fv.

figure 1. In view of the effect of age on the A:G ratio in foals 3 to 6 months old, this group was excluded from the summary. According to the averages for the seasons, the vernal decline in total serum-protein level is due primarily to a reduction in the albumin fraction. The averages for each month, in many instances representing too few cases for definite conclusions, indicate that this fall in albumin precedes a secondary reduction in globulin. There is, therefore, an initial reduction in the A:G ratio as the total protein begins to recede after February, with a minimum ratio during March and April (1.1), and subsequently a return to the February level (1.4) by May. A similar trend was not encountered with the autumnal fall in total protein, which may be due in some measure to the fact that a large number of the group on which fractionation was conducted came late in November 1939, after cold weather had become well established. The total protein levels in these late November cases were distinctly within the range of the December cases; specimens collected early in November, and particularly during 1938, when the weather was mild in southeastern Pennsylvania and eastern Maryland, revealed a total protein average slightly lower than 6 Gm. per 100 cc. of serum.

A similar trend was observed in the level of total calcium. The average calcium in 24 normal horses examined in May was 11.61 mg. per 100 cc. of serum, a minimum following a steady reduction from an average of 12.55 observed in February (figure 1). Comparing the curves for total calcium and total protein, it may be noted that the agreement between the trends evident during the first half of the year is not maintained after midsummer. From a high level in July (12.51) the average calcium sinks to 12.02 in August, to rise gradually to another high point in November (12.52), while the proteins remain relatively constant until October. The reason for this discrepancy is not clear.

Further study of the curves in figure 1 leads to the conclusion that the seasonal fluctuations in total calcium and protein are interrelated. Ca^{++} averages in this series show little variation except the fall and rise in May and June and a second sharp rise in November. In no case, however, is the variation great enough to explain the changes in total calcium. Presumably, these variations reflect modifications in the nondiffusible calcium (*i.e.*, calcium proteinate).

Phosphorus levels in mature horses of both sexes, including pregnant mares, showed a seasonal tide similar to that ob-

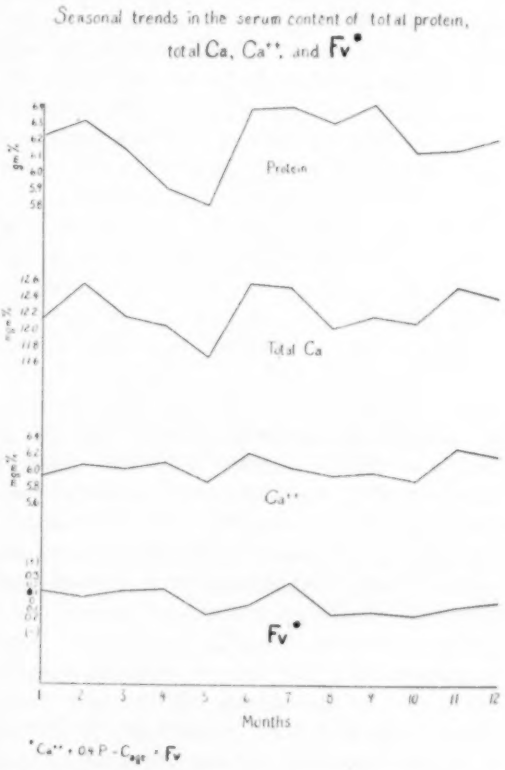


Fig. 1. Seasonal trends in the serum content of total protein, total Ca, Ca^{++} and Fv. These data were obtained from males and nonpregnant females of all ages, excluding only foals less than 3 months old from the averages for protein and Ca^{++}. The monthly distribution of averages, together with the number of cases included in the summaries, is presented in table II. In the case of Fv, only normal animals free from any evidence of pathology, local or systemic, were tabulated (total 161). The other curves include results from cases of clinical nature, where there was no reason for, or evidence of, disturbance in the serum component involved. Extreme values were omitted from all compilations. The total numbers of cases summarized in these averages are: total protein, 278; total Ca, 290 (including young foals); Ca^{++}, 276. These studies extended over a two-year period ending in January 1940.

served in children by Hess and Lundagen.[59] These investigators reported, however, that the tide occurs only in young children; in adults the blood phosphorus is little affected by seasonal changes. Averages by month of the inorganic phosphorus in horses 5 years old and over revealed a consistent rise from a minimum of 2.8 mg. per 100 cc. in March to a peak of 3.9 in July. Thence it gradually receded to 3.3 by September, where the average remained until winter, when further reduction was encountered. During the winter and early spring season (December to April) the average serum phosphorus in 46 tests was 2.9, as compared with 3.4 during the summer (June to September), the average from 38 determinations. Pregnant and post-parturient mares showed the same tide as the males and nonpregant females.

Feeding.—Early in the course of this study it was noticed that animals from different farms show distinct differences in blood chemistry. Considering the possible factors, it was concluded that these differences are the result of variations in the feed. Unfortunately, it was not possible to obtain accurate data on the feeds supplied these horses, in the absence of controlled feeding and chemical analyses of the foodstuffs. It is possible, however, to compare in a general way the farms where the horses were stabled and pastured. From the standpoint of soil composition, pasture growth, quality of feeds purchased and the use of various supplements in the ration, an approximate basis was established for comparing the farms as regards mineral intake and general nutrition afforded the horses.

Some of the farms are favored geographically with rich limestone soil. Others require periodic fertilization to maintain a comparable supply of calcium in the crops. On farms in the latter position, where the available mineral of the soil has not been supplemented frequently, the crops tend to be lower in calcium and phosphorus than where the soil is either naturally adequate or the deficiencies have been corrected.

[59]Hess, A. F., and Lundagen, M. A.: A seasonal tide of blood phosphates in infants. J.A.M.A., lxxix (Dec. 30, 1922), p. 2210.

It was found, in general, that the horses on farms affording superior feeding conditions were uniformly higher in blood calcium than the horses from farms inferior in this respect. Average figures in extreme cases differ from 1 to 2 mg. per 100 cc. of serum. This has appeared as the greatest individual source of normal variation in the calcium. The deviation was most marked in young animals past the suckling age and in brood mares. Suckling foals did not seem to be influenced, nor was the effect so distinctly evident in mature males and nonpregnant females. Inorganic phosphorus did not appear definitely altered, although average figures obtained on horses from the superior farms tend to be slightly higher than those from the less favored animals. No comparable variation was observed in the total protein level of the serum; but the A:G ratio was diminished in animals subject to the combined effects of suboptimal feeding and intestinal parasites.

Pregnancy.—Mares in foal and mares recently delivered showed distinct variations from the nonpregnant mares, especially in the serum proteins. Pregnant mares uniformly revealed lower albumin levels, together with such an excess of globulins that the total protein was higher than in nonpregnant mares (table I). Accordingly, the A:G ratio was considerably lower, averaging 0.8, as compared with 1.3, the average for males and nonpregnant females.

From the histories of the 40 mares examined, collected by questioning the owners or attendants and studying the records of the animals where available, no evidence could be obtained to suggest a definite connection between these blood-chemistry findings and the stage of pregnancy, number of former foals, or health of the foals produced by these mares. Available evidence was both meager and contradictory. It was found, in the few cases followed after foaling, that the mares which were not bred again, or having been bred failed to conceive, continued for several months with the protein levels of the pregnant mares.

It appears, from the limited information obtained on seasonal fluctuations in pro-

teins, that the conclusion derived on males and nonpregnant females may be applied to this group, with a modification. During February, March and April the averages were lower than the average for the entire group, but a high average protein level appeared in May. Since these mares foaled during April and May, it was assumed that the physiologic processes attending parturition may offset any further tendency toward a seasonal reduction in the serum-protein concentration at that time.

TABLE III—Factors for Determining Serum Protein Equivalent at Albumin: Globulin Ratio of 1.8

A:G	FACTOR
0.1	0.81
0.2	0.83
0.3	0.85
0.4	0.87
0.5	0.89
0.6	0.90
0.7	0.92
0.8	0.93 (a)
0.9	0.94
1.0	0.95
1.1	0.96
1.2, 1.3	0.97 (b)
1.4	0.98
1.5, 1.6	0.99 (c)
1.7, 1.8, 1.9	1.00 (d)
2.0, 2.1	1.01
2.2, 2.3, 2.4, 2.5	1.02
2.6, 2.7, 2.8, 2.9	1.03
3.0	1.04

These factors are based on the alkali-combining powers of albumin (*f*-prot. 0.273) and globulin (*f*-prot. 0.189) as determined on the purified proteins.[43] In cases where the A:G ratio is established by fractionation, the figure for total protein (in Gm. per 100 cc. of serum) is multiplied by the appropriate factor to compute the equivalent protein concentration at A:G 1.8. This product is applied in using the nomogram (fig. 2) for estimating Ca++.

In the absence of pathology altering the ratio, the fractionation may be omitted with negligible error, if the following corrections are assumed:

(a) pregnant mares, 0.93

(b) mature males and nonpregnant females, 0.97

(c) foals, 3 to 6 months old, 0.99

(d) foals, less than 3 months old, no correction.

The total calcium, however, showed a gradual decline from the February level (11.9) through May (11.5), whereupon the average rose (to 12.0), following the trend observed in males and nonpregnant females. On the basis of the small number of reports obtained throughout the remainder of the year, it appears that the calcium level continued to rise in the blood of brood mares during the summer, to reach a maximum during August (12.8). With the return of cooler weather these high levels gave way to averages in the neighborhood of 12 mg. per 100 cc., which then remained fairly constant until the time of the downward trend in spring.

These observations are concordant with the opinion that there is a definite tendency toward hypocalcemia during the final stage of pregnancy, when the fetus is making strong demands on the dam for calcium to promote active ossification. It is at this time that dietary deficiencies are felt most sharply. Brood mares on the farms where the feed was considered inferior showed a tendency toward low serum-calcium levels at all times, and particularly during the spring, prior to the foaling period. On farms where adequate measures had been taken to maintain the feed at a high calcium content, the tendency toward a prepartum decline in blood calcium was less marked, and during the remainder of the

TABLE IV—Values for the Constant C_{age} for Horses of Different Ages

AGE (MO.)	C_{age}	AGE	C_{age}
1	9.5	12-14 mo.	8.2
2	9.3	15-17 mo.	8.1
3	9.2	18-20 mo.	8.0
4	9.0	21-23 mo.	7.9
5	8.9	2 yr.	7.8
6	8.8	2½ yr.	7.7
7	8.7	3 yr.	7.6
8	8.6	3½ yr.	7.5
9	8.5	4 yr.	7.4
10	8.4	5 yr.	7.3
11	8.3	Over 5 yr.	7.2

The above constants are used in determining Fv in the equation, $Ca^{++} + 0.4\ P - C_{age} = Fv$, where Ca^{++} and P represent, respectively, the Ca^{++} (calculated) and total inorganic P of the serum, both expressed as mg. per 100 cc.

Nomogram for Calculating Calcium Ion Content of Blood

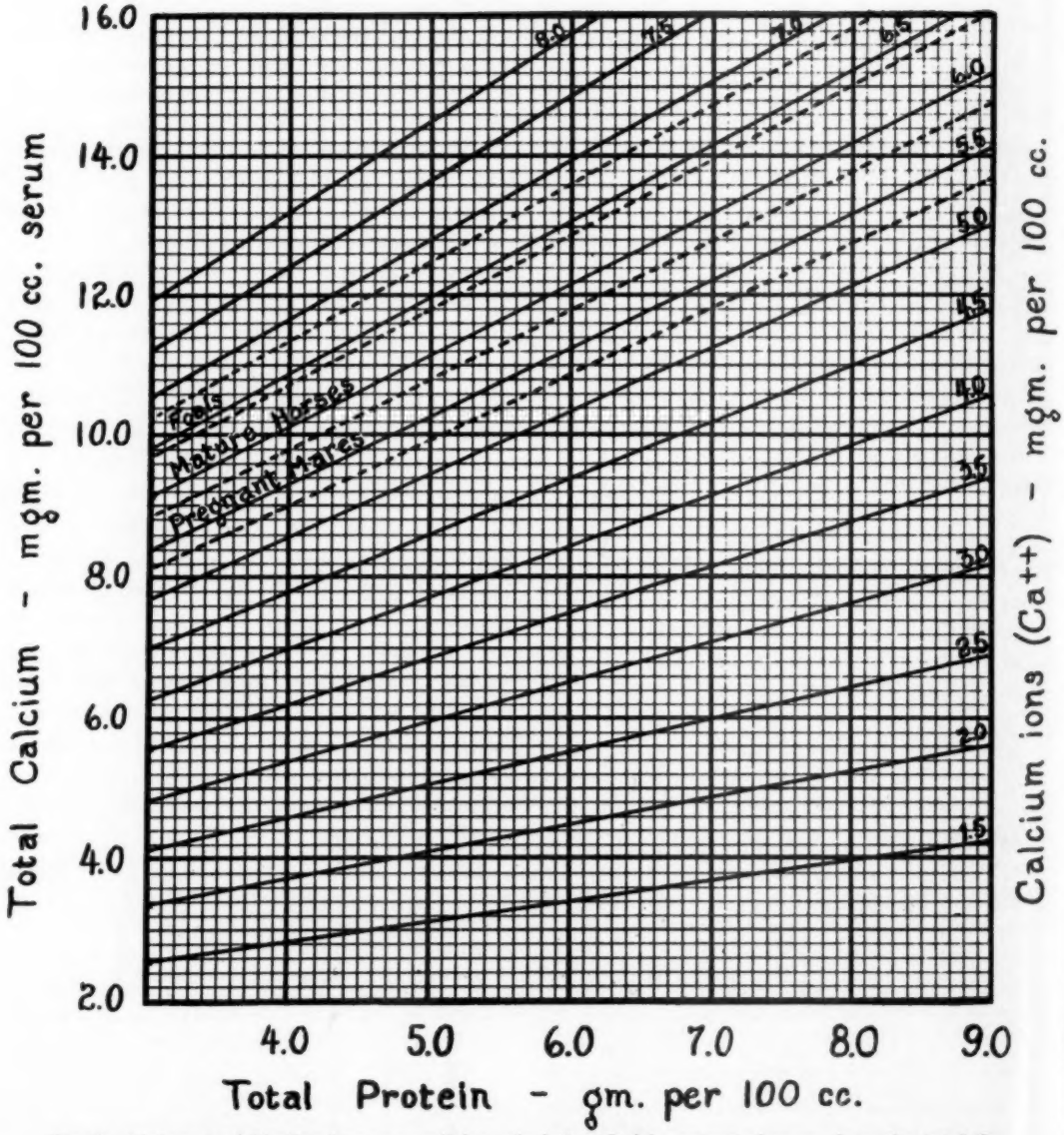


Fig. 2. McLean and Hastings' nomogram[11] for calculating Ca^{++} content of serum from the total Ca and total protein levels. The relationships expressed are based on an equation in which the A:G ratio of the proteins is assumed to be 1.8, the average normal in man. In view of the different ratios encountered in this study, a protein correction is desirable in the case of mature horses, and especially in the case of pregnant mares.

To calculate the Ca^{++} level in a specimen, read between the diagonal lines of the nomogram the point at which the horizontal line, representing total Ca, intersects the vertical line, representing equivalent total protein (see table III). The broken lines indicate approximate ranges for young foals, mature horses and pregnant mares (see table I).

TABLE V—Plasmapheresis Experiment

Exam.	Date (1938)		Ca (Mg. %)	Ca++ (Mg. %)	P (Mg. %)	Protein (Gm. %)	A:G	Ca++ + 0.4 P	Fv*
a	5-4	First examined: lame	10.4	5.3	2.2	5.7	1.4	6.2	—1.0
b	8-1	Removed 7 L. blood	12.4	5.9	2.2	6.5	1.2	7.0	—0.2
c	8-2	Removed 7 L. blood; returned 7 L. corpuscle-saline	11.3	5.6	1.7	6.0	1.1	6.3	—0.9
d	8-3	Removed 7 L. blood; returned 7 L. corpuscle-saline	11.2	5.8	1.2	5.5	1.1	6.3	—0.9
e	8-4	Removed 7 L. blood; returned 7 L. corpuscle-saline	10.0	5.5	2.0	4.9	1.2	6.3	—0.9
f	8-11	Neck swollen; poor appetite; temperature normal	11.2	5.5	3.0	6.1	0.8	6.7	—0.5
g	8-12	Neck swollen; better appetite	11.3	5.5	4.7	6.3	0.8	7.4	+0.2

*$Ca^{++} + 0.4\ P - C_{age} = Fv$.

Thoroughbred gelding, 7 years old: chronically lame with a diagnosis of rarefying ostitis of the distal sesamoid of the front legs (navicular disease). From the time of first examination (5-4), and for the following two weeks, 4 dr. of syrup of calcium lactophosphate was added daily to the ration. The daily procedure in this experiment (8-1 to 8-4) was to collect a specimen of jugular blood for analysis, then remove 7 liters of blood, which was defibrinated promptly. The defibrinated blood was set aside 20 to 24 hours, after which the supernatant plasma was siphoned off from the corpuscles and replaced with an equal volume of 0.9 per cent NaCl solution. This corpuscle-saline solution was warmed to body temperature and returned to the animal's circulation. The experiment was interrupted upon the fourth bleeding (8-4) by a marked phlebitis and swelling of the entire lower surface of the neck; for a few days poor appetite and immobility of the head and neck gave evidence of discomfort, but there was no rise in the body temperature.

TABLE VI—Effects of Variations in the Feed on the Blood-Chemical Composition in Six Thoroughbred Foals

Date (1938)	Feed			Ca (Mg. %)	Ca++ (Mg. %)	P (Mg. %)	Protein (Mg. %)	A:G	Ca++ + 0.4 P	Fv*
10-13				11.8	5.6	6.7	6.5	1.3	8.3	—0.4
11-14	LIMED PASTURE			12.7	6.5	4.7	5.9	1.3	8.4	—0.3
12-5	LIMED PASTURE		BRAN CORN	13.0	6.5	5.4	6.1		8.7	+0.1
(1939) 1-15	LIMED PASTURE	COD LIVER OIL	BRAN CORN							
2-6	LIMED PASTURE	COD LIVER OIL		11.9	5.9	6.1	6.0		8.4	0
4-19	LIMED PASTURE			12.8	6.5	3.7	6.0		8.0	—0.3

*$Ca^{++} + 0.4\ P - C_{age} = Fv$.

All figures represent averages obtained from the six foals examined on each of the above dates.

Weaning had been started on this group of foals (5 to 8 months old) at the time of the first examination. During the experiment they all were fed good hay (mixture of clover, alfalfa and timothy) and oats, supplemented as described. The pasture on which they grazed during the period ("limed pasture") was treated with lime several months previously and was neither cut nor used for grazing during the interval, resulting in a luxuriant crop of mixed grasses. The pasture used before and after this period was not so treated, and had been in almost constant use for years.

The ration supplied the foals was varied as follows: Oct. 14, first grazed on limed pasture; Nov. 15, bran and corn added to feed; Dec. 5, cod liver oil added to feed and use of bran and corn discontinued; Jan. 16, removed foals from limed pasture and returned them to old pasture; Feb. 7, returned to limed pasture; Apr. 19, cod liver oil continued to end of the study.

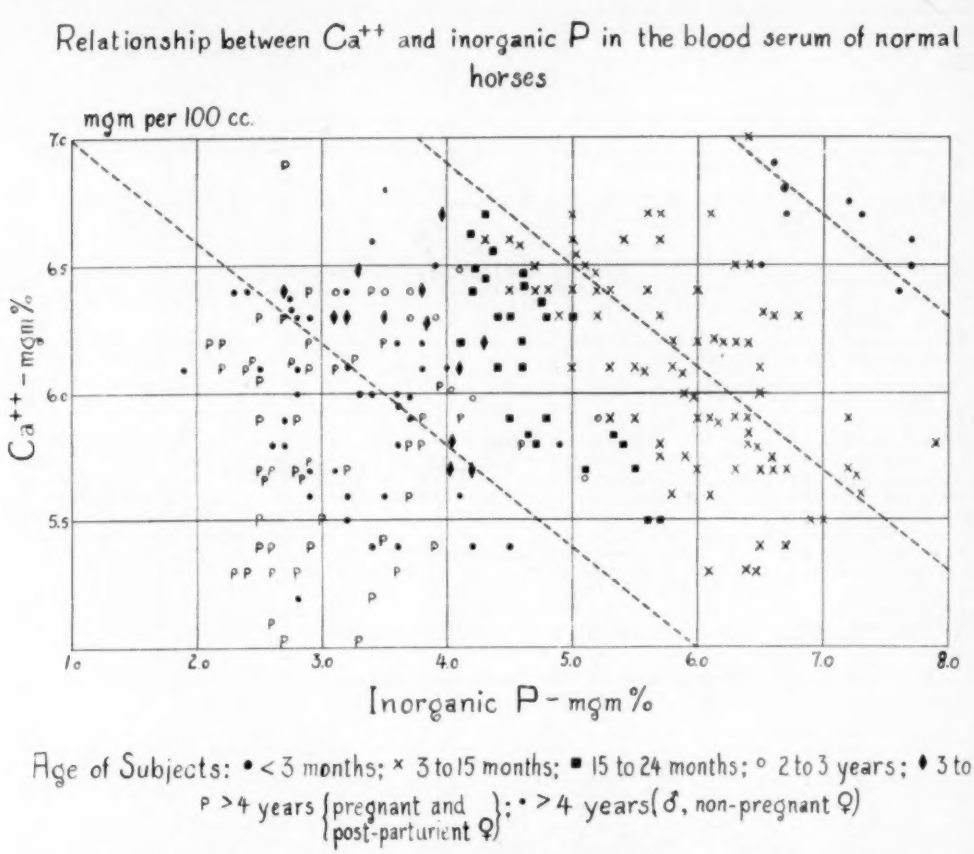


Fig. 3. Relationship between Ca^{++} and inorganic P in the blood of normal horses. The figures for Ca^{++} (calculated) and inorganic P, obtained from 239 examinations on 187 different normal horses, were collated with regard for the age of the subject, and pregnancy or recent parturition. The effect of age on the P level is indicated by the position of the various age groups on the abscissae. The reciprocal nature of variations in Ca^{++} and P appears in the tendency of the points plotted for a limited age group to follow a line expressing the equation, $Ca^{++} = C_{age} - 0.4\ P$, where Ca^{++} and P are expressed as mg. per 100 cc., and C_{age} is a constant varying with the age of the subject. The diagonal broken lines indicate a few of these age constants.

year the levels were as high as those of the nonpregnant mares.

Inorganic phosphorus showed little alteration that could be correlated with pregnancy. As indicated in a preceding paragraph, a seasonal tide was encountered among the brood mares. The average phosphorus was slightly lower in pregnant mares during the first half of the year, as compared with the findings in males and nonpregnant females, while during the second half of the year, the former showed slightly higher phosphorus levels.

CORRELATION OF ANALYTIC FINDINGS

In this application the proteins may be regarded as a primary variable, since it is not conceivable that alterations in either calcium or phosphorus can influence the protein concentration. In normal animals the proteins were found to vary from 5 to 7 Gm. per 100 cc. of serum, under the influence of age and seasonal changes. This maximum frequently was exceeded in normal pregnancy as well as in the presence of pathology accompanied by systemic reactions. (See tables I, VIII and X.)

In view of the different base-combining powers of the albumin and globulin fractions in horse blood serum, as determined by Van Slyke, Hastings, Hiller and Send-

roy,[60] variations in the level of total proteins are partly offset in many cases by alterations in the A:G ratio. It is evident that the apparent excesses of total serum protein in pregnancy and cases of pathology, recorded on a weight basis (*i.e.*, grams per 100 cc. of serum), for this reason are out of proportion with the relative increase in molecular concentration (*i.e.*, millimols per kilogram of water). To overcome this source of error, as well as to gain additional information on the serum proteins, we have studied the A:G ratio in the majority of our normal subjects and most of the clinical cases suspected of a systemic reaction to disease.

Total calcium of the serum, on the other hand, has been shown to depend on both the proteins and the inorganic phosphorus. The nondiffusible calcium fraction varies directly with the proteins, and Ca^{++} inversely with the phosphorus. Reflected in the level of total calcium, these variations in fractions of the element sometimes lead to alterations beyond the normal range, to be mistaken for primary disturbances in the blood calcium. Conversely, significant variations in calcium go unrecognized in the shadow of alterations in the proteins and phosphorus.

To coördinate these data we followed the formula of McLean and Hastings for calculating Ca^{++} concentration from the total calcium and total protein levels in the specimen. In practice a nomogram (fig. 2) was employed for this purpose,[61] correcting

[60] Van Slyke, D. D., Hastings, A. B., Hiller, A., and Sendroy, J. J.: Studies of gas and electrolyte equilibria in blood. xiv The Amounts of Alkali Bound by Albumin and Globulin. J. Biol. Chem., lxxix (Oct. 1928), p. 769.

[61] McLean, F. C., and Hastings, A. B.: Clinical estimation and significance of calcium-ion concentrations in the blood. Amer. J. Med. Sci., clxxxix (May 1935), p. 601.

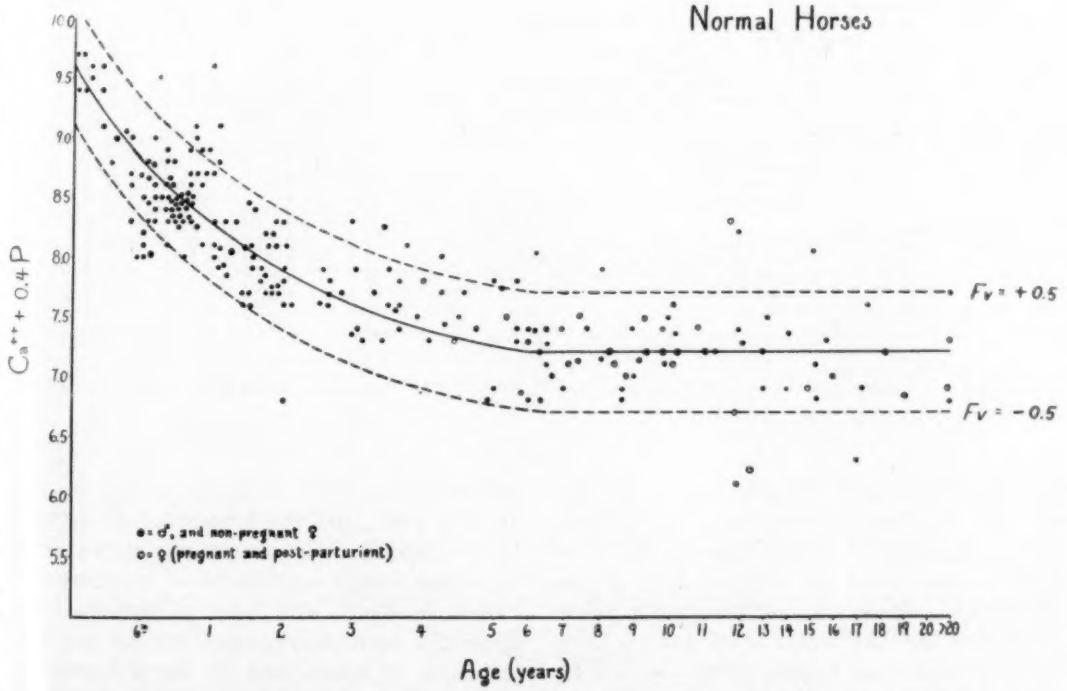


Fig. 4. Relationship between Ca^{++} + 0.4 P and age in the serum of normal horses. The findings in normal horses (all ages, both sexes, pregnant and post-parturient females) expressed as Ca^{++} + 0.4 P are aligned on an age basis. The curve in solid line represents the average normal value identified as C_{age}, a constant varying with the age of the subject up to 6 years. The curves in broken lines indicate the assumed limits of normal variation. Expressed as the following formula, Fv normally equals 0.0 (s.d. 0.3):

$Ca^{++} + 0.4\ P - C_{age} = Fv.$

The assumed normal limits in this graph are + 0.5.

TABLE VII—Summary of Findings in Cases with Bone Involvement

	Acute			Subacute			Chronic		
	Low	s. d.*	High	Low	s. d.*	High	Low	s. d.*	High
Ca^{++} cases	6	1	0	7	4	3	8	12	3
Ca^{++} cases	4	3	0	5	6	3	11	10	2
P cases	4	3	0	2	12	0	3	14	6
Fv‡ cases	6	1	0	8	5	1	3	19	1
%	86			57			13		
Ca^{++} mean difference†	−0.6		0	−.3		+0.4	−0.4		+0.2
P mean difference†	−2.2		0	−0.5		0	−0.4		+0.5
Fv‡ mean		−1.3			−0.4			−0.1	
maximum		−0.2			+0.9			+0.9	
minimum		−2.5			−0.9			−1.2	
Total cases		7			14			23	

†Mean of values obtained in the groups outside the s.d. range; *i.e.*, mg. per 100 cc. serum.

*S.d. range, as indicated in table I; in the case of Fv values the range +0.5 to −0.5 was used, all findings within this range regarded as normal; cases with findings above and below these ranges are classed as "high" and "low" respectively.

‡$Ca^{++} + 0.4\ P - C_{age} = Fv$.

TABLE VIII—Typical Records from Cases of Bone Involvement

No.	Age	Date	Condition	Ca (Mg. %)	Ca^{++} (Mg. %)	P (Mg. %)	Fv*	Proteins	A:G
	Acute inflammation								
240	3 mo.	6- 8-39	Fractured radius 3 weeks ago; poorly healed	*11.2*	*5.3*	*3.5*	−2.5	*6.6*†	*1.0*
267	6 mo.	12- 6-39	Rickets; both stifles enlarged	*11.5*	*5.8*	*3.6*	−1.6	5.7	
31	20 yr.	3-22-38	Acute general arthritis	*9.5*	*4.4*	3.2	−1.5	*7.4*†	*0.5*
225	1 mo.	4-19-39	Acute arthritis; hock enlarged from birth	*11.5*	*6.8*	*3.7*	−1.2	*4.2*	
		7- 7-39	Joints normal, but poor in flesh	*14.3*†	*6.8*†	7.6	+0.9	6.5	
	Subacute inflammation								
239	3 mo.	5-19-39	Enlarged ankles	*11.4*	*5.8*	6.9	−0.7	5.6	1.5
106	3 mo.	8-25-39	Enlarged ankles	*11.4*	5.9	6.1	−0.7	5.4	1.5
246	2 yr.	7-13-39	Periostitis ("bucked shins")	*13.5*†	6.4	3.6	0	6.6	1.6
79	2 yr.	5-20-38	Enlarged fetlock and ankle joint	*11.4*	*5.7*	4.1	−0.5	5.8	1.5
	Chronic inflammation								
75	8 yr.	5-17-38	Spavin; poor general condition	12.0	5.7	2.9	−0.3	6.8	0.9
289	1 yr.	5-14-40	Extreme deformity of spine since birth	12.5	6.3	4.6	−0.1	6.1	
73	5 yr.	5-12-38	Ringbones; general condition fair; lame	*11.5*	*5.5*	3.2	−0.5	6.5	1.0
		7- 2-38	Sound; general condition better	12.3	6.3	3.2	+0.3	5.9	1.2
180	20 mo.	12-16-38	Splints	11.8	*5.5*	*5.7*†	−0.1	6.7	

*$Ca^{++} + 0.4\ P - C_{age} = Fv$.

†In excess of normal range; other figures *in italics* without (†) represent values lower than the normal minimum.

for variations from the standard A:G ratio of 1.8. For convenience these corrections were accomplished by multiplying the total protein concentration, expressed as grams per 100 cc. of serum, with a factor calculated for the particular A:G ratio (table III). The product, representing the equivalent molecular concentration of protein at A:G 1.8, was applied to calculate Ca^{++}.

The Ca^{++} values thus obtained then were plotted against the phosphorus findings on the same specimens from a series of normal horses. Since a large part (about 90%) of the phosphorus is in the diffusible state, and the proportion of diffusible to nondiffusible phosphorus shows little variation under conditions of considerable range in Ca^{++} and total phosphorus, no allowance seemed necessary for the possible influence of alterations in the diffusibility of the phosphorus present. It was found that, within a limited age group, Ca^{++} varies inversely with the concentration of phosphorus (fig. 3). This relationship may be expressed as:

$Ca^{++} + 0.4\ P = C_{age}$ (a constant for

an age group), (both Ca^{++} and P expressed as milligrams per 100 cc. of serum).

In order to evaluate this constant, C_{age}, for each group, the results were plotted as shown in figure 4, and a smooth curve drawn to represent theoretical normal values. Knowing the constant for any given age (table IV), it is possible to compare the data obtained from one group with those obtained from another, applying the following formula:

$$Ca^{++} + 0.4\ P - C_{age} = Fv$$

The data in table V illustrate the procedure.

It is interesting to compare this equation with that of Peters and Eiserson.[50] Rearranging the terms (b) from the original order (a) emphasizes the similarity between their findings from mature humans and our data from mature male and nonpregnant Equidae (c):

$$\text{Total Ca} = -0.225\ P + 0.556\ \text{protein} + 7 \quad \text{(a)}$$

Man: $(\text{Total Ca} - 0.556\ \text{protein}) + 0.225\ P - 7 = 0$ (b)

Horse: $Ca^{++} + 0.4\ P - 7.2 = 0$ (c)

The main differences lie in methods of correlating calcium and protein figures, and the factors ascribed to the phosphorus portion. It seems significant that the numerical value of both equations is about the same in both species, in view of the relatively higher calcium and lower phosphorus levels in horse blood.

The empirical formula is to be regarded

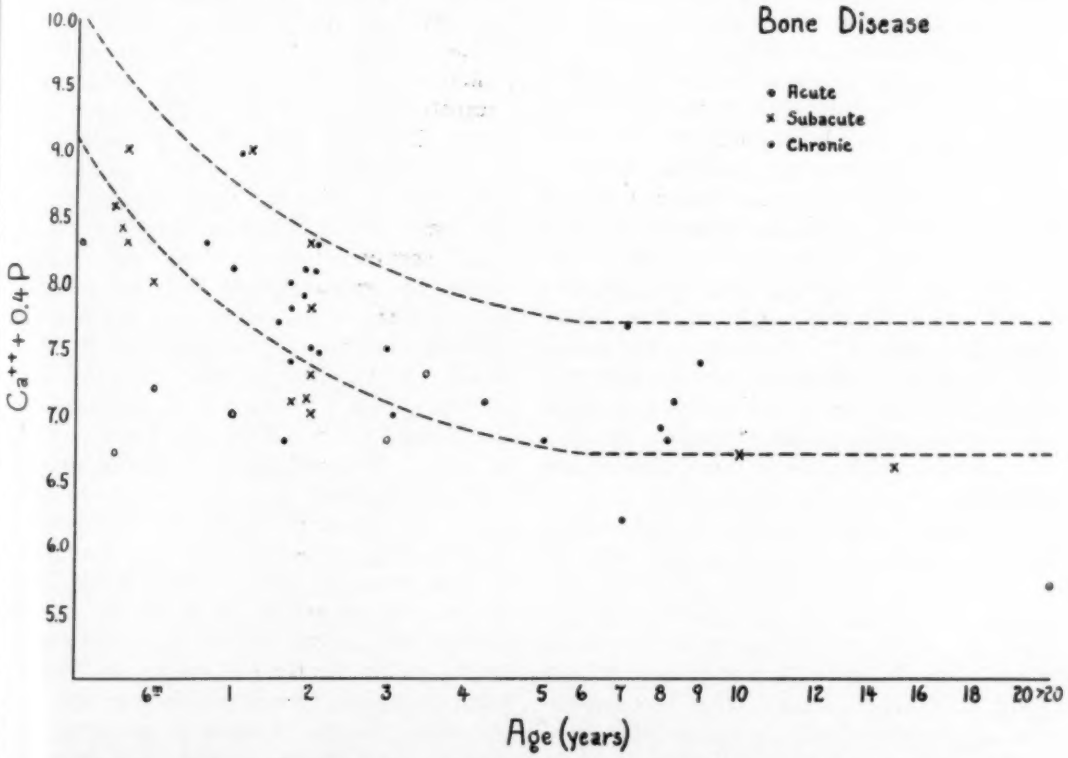


Fig. 5. Distribution of results from cases with symptoms of bone disease. These cases have been classified as acute, subacute and chronic, according to symptoms manifest at the time of examination and history of the disturbance. These findings are summarized in table VII, and illustrated by the Abbreviated case records in table VIII.

It is apparent from the distribution of results on this graph that acute bone lesions (acute arthritis, acute ostitis) are associated with marked reduction in Fv, indicated by the frequence of values considerably below the assumed normal minimum. This reduction is less marked in connection with subacute lesions, although over one half of the cases in this group fall below the normal minimum. The findings in chronic cases indicate little abnormality.

as a clinical approach to the interpretation of blood findings in cases of suspected primary disturbance in the calcium and phosphorus. By reference to the averages obtained on normal animals, fluctuations in these elements in a case in question may be regarded as either (1) primary or (2) secondary to variation in the complementary element or the proteins of the blood. This decision is based on how closely the sum of the elements, calculated in this manner, approaches the average for normal animals; that is to say, how closely Fv approaches 0. Speculating on the possibilities suggested, it may be inferred that when Fv is a positive value, the blood contains Ca and P in excess of the usual concentrations in equilibrium with the precipitates in the bones. The opposite conclusion is implied by a negative figure. In both instances the degree depends on the magnitude of the difference.

A correlation between variations in the calcium and the proteins of serum is suggested by the comparable trends in their seasonal fluctuations, portrayed in figure 1. The Ca^{++} figures on the same subjects failed to show the same trend, except for the low average in May, when a minimum of 5.86 mg. per 100 cc. was observed, followed by a sharp rise to 6.21 in June. Apart from this, the Ca^{++} averages reflect indifference to the curves for calcium and protein, remaining practically constant from January to April, notwithstanding the distinct downward trends in total calcium and protein.

The same sharp reduction in the average findings obtained in May was observed for Fv, the value of which was relatively constant during the earlier months of the year, and again from August to December. From a minimum in May it rose to a high point in July, in accord with the conception of a seasonal fluctuation in solar irradiation influencing the utilization of phosphorus, and possibly of calcium also. This may be compared with the trend in phosphorus observed in the mature horses, although it is to be remembered that the averages under Fv include several times more figures from young than from mature animals. It would be impossible to compare on a seasonal basis the phosphorus findings from young animals of various ages without due allowance for difference in age. The formula suggested eliminates both the effect of age and the influence of variations in calcium and protein. It is interesting to note, in this connection, that the peak of Ca^{++} concentration occurred in June, and was not the result of a progressive rise, but, rather, appears as an erratic variation. On the other hand, both the nature of the seasonal curve for Fv and the time of its peak agree with the curves for inorganic phosphorus obtained by Hess and Lundagen with children, and by ourselves with mature horses.

The correlation was studied further in two experiments. In one, the proteins were reduced gradually by plasmapheresis. Each day for four days, 7 liters of blood was removed from a horse weighing approximately 400 kg. This blood was defibrinated promptly and placed in a refrigerator overnight. The next day, after 20 to 24 hours in the refrigerator, the serum was removed and replaced with an equal volume of 0.9 per cent NaCl solution (physiological). This corpuscle suspension was mixed, warmed to approximately body temperature and returned to the circulation of the horse following the removal of another 7 liters of blood. At the end of four days the level of total protein in the serum had been reduced from 6.5 to 4.9 Gm. per 100 cc. (table V, b.-e.). The reduction of serum protein was accompanied by a loss of phosphorus and a progressive decline in the calcium. It is notable, however, that the decline in calcium was not strictly comparable with the protein reduction, as shown by a temporary rise in the Ca^{++}. These changes, considered in the light of reciprocal fluctuations, indicate alterations in the calcium secondary to the changes in proteins and phosphorus. The relatively constant value of Fv points to this conclusion.

The second experiment was carried out with six foals, ranging from 5 to 8 months old at the beginning of the study (table VI). Upon transferring them from a sparsely covered paddock to a pasture of

thick grass, well limed several months before and on which there had been no grazing, they showed a marked rise in serum calcium, accompanied simultaneously by reduction in the proteins and phosphorus. It is evident that the Ca^{++} rose sharply during this period. The reduction in phosphorus was comparable with this rise in Ca^{++}, as indicated by the lack of significant variation in Fv.

At the time of the second blood examination these foals were treated with intestinal anthelmintics. A month later, improved in appearance and evidently in better general condition, they showed a slight increase in Fv, due to a rise in phosphorus without a reduction in the Ca^{++} levels. When the foals were removed from the limed pasture, the calcium levels fell, with a rise in the phosphorus compensating largely for the Ca^{++} reduction. This reduction in calcium occurred in spite of the liberal use of alfalfa hay and cod liver oil during the period of return to the original pasture. In the opinion of the trainer, the condition of these animals suffered somewhat from the change; they were not as vigorous nor as sleek in appearance as they were when on the limed pasture. Upon return to this pasture, outward signs of improvement were perceptible, while analysis showed calcium in the blood to have risen to the former high levels. This rise was accompanied by a compensatory reduction in phosphorus, with but slight rise in Fv.

PATHOLOGY OF THE BONES

The cases reviewed in this division (chiefly arthritis, ostitis and exostosis) are typical of routine veterinary practice. Diagnosis was based on the history together with the symptoms presented at the time of examination, in many instances supported by roentgenologic studies. In some cases, such as fractures or other sudden injuries which were recognized immediately after they occurred, definite information could be obtained on the duration of the disturbance; in others the duration could not be established. Rickets and progressive rarefying ostitis (navicular disease) provide examples of the more insidious type; these cases usually go unrecognized until the bone-weakening processes have been in effect a considerable time.

The majority of the cases studied in this division evinced symptoms of local inflammation in parts convenient for observation and palpation (*e.g.*, legs and feet). In these the following criteria were employed in a general way for differentiating the various stages of inflammation:

Acute: Pain most marked (lameness, and increased sensitiveness to applied pressure).
Heat (area warmer to touch than the surrounding parts).
Swelling.
Subacute: Pain less marked than in the acute stage.
Heat usually absent.
Swelling as great or greater than in the acute stage.
Chronic: Pain and heat entirely absent (lameness may persist due to mechanical interference in movement of the bones and their attachments).
Swelling remaining, and may be permanent.

Records to date include 7 cases of acute nature, 14 subacute and 23 chronic, a total of 44. The results of blood analyses are collated in table VII. To illustrate the general trend of results, typical cases from each of these groups are reviewed in table VIII. The data represent findings preliminary to any treatment capable of affecting the blood calcium and phosphorus. The effects of treatment, such as mineral and vitamin D supplements in the feed and parenteral injections of calcium salts, are summarized in another division of this report.

Serum proteins were unaffected in cases of uncomplicated bone lesions. Notable exceptions to this, however, were encountered in three of the acute cases, cited in table VIII. Two of these were essentially cases of acute inflammation in which the effects of the inflammatory process had involved protein regulation (Nos. 240 and 31). The third was a newborn foal with a con-

genital joint involvement complicated by malnutrition (No. 255). It is interesting to find in this last case that the Ca^{++} did not vary between the first and second examinations, although total calcium increased from 11.5 to 14.3 mg. per 100 cc. The rise in total calcium was attributed to an increase in the nonionic fraction (*e.g.*, calcium proteinate) in proportion to the variation in protein level. The improvement in Fv during this period was the result of a rise in phosphorus.

Acute Bone Lesions.—This group comprises two cases of spontaneous joint enlargement in foals, three cases identified as acute arthritis in older horses, and two fracture cases. In all of these the diagnosis indicated active inflammation in the bones, and it is apparent from a study of table VII that the blood was inclined to be low in calcium and phosphorus. Coördinating the findings according to the formula suggested, the results in terms of Fv showed six out of seven (86%) to be low in this respect, the group averaging —1.3. It is significant, perhaps, that no case in this group was high in Ca^{++}, phosphorus or Fv; in fact, Fv was in all cases a negative value, falling within the normal range in only one instance.

Subacute Bone Lesions.—This subdivision contains seven cases of subacute arthritis in young horses, three cases of periostitis (bucked shins) in 2-year-olds, and four cases of subacute ostitis (osselets and splints). From the symptoms and histories available, they were classed as subacute. While a small but signficant number revealed low blood calcium, only two out of 14 were low in phosphorus. On coördination of the results, however, it was found that eight out of 14 (57%) showed a significant reduction in Fv, and the average for the group was —0.4.

Chronic Bone Lesions.—In this group are found twelve cases of various exostoses (splints, ringbones and spavins), six of chronic arthritis, four of chronic ostitis (navicular disease and proximal sesamoiditis), and one case of calcified supraspinatous tendon and bursa. Although half of these cases were low in Ca^{++}, the levels for inorganic phosphorus were either normal or high (table VII); and since the cases with low Ca^{++} level were usually found with high phosphorus, very few (three out of 23) showed signficant reduction in Fv. The average of the group for Fv (—0.1) was almost identical with that of normal animals.

Comparing the results from three groups with bone lesions, the most striking conclusions concern the trend in Fv and the proportional variation in the individual elements, Ca^{++} and phosphorus. Although the number of cases in each group is too small to permit more than tentative conclusions, the general trend seems definite enough. The levels of these elements, as measured by the formula expressing the hypothetical Fv, are distinctly low in the acute cases, but normal in the chronic ones. This is illustrated by both the numbers of cases with significantly reduced Fv, and the averages for the groups. Of the two elements involved, phosphorus seems to have the greater influence in this trend. The number of cases with a low Ca^{++} [below the range for standard deviation from the mean (see table I)] varies but little among the groups, while phosphorus figures vary in a definite order. The group of acute cases reveals four out of seven with low phosphorus, and no high ones; among subacute cases two out of 14 are low; while three out of 24 of the chronic cases show low phosphorus, and six are high.

It may be added that while the proportionate number of acute cases with low Ca^{++} does not exceed greatly that of the subacute and chronic groups, the average difference is somewhat greater. This is evident from the second series of figures in table VII, as is also a very wide difference in the phosphorus in the acute group, in comparison with the others. Apparently both these elements are inclined to be diminished in the blood of acute bone cases, with the phosphorus affected more markedly than the calcium. In the subacute and chronic cases these effects are progressively less pronounced. In fact, in the latter group, apart from a tendency toward mild

reductions in calcium, and a compensatory rise in phosphorus, there appears little deviation from the normal.

PATHOLOGY OF THE SOFT TISSUES

The cases studied in which bone disturbance was not involved have been subdivided, as far as possible, into respiratory, gastrointestinal, nervous and miscellaneous. The last subdivision includes those which could not be classified in one of the others, and not sufficiently numerous to form a separate class. Cases of spontaneous epistaxis (bleeders) are considered specially. Since the above classification is based on opinions derived from clinical symptoms, and each class may cover a diversity of physiological disturbances, the conclusions are to be regarded as general diagnostic trends.

Respiratory.—A total of 13 examinations was made on ten different cases, diagnosed bronchitis (5), pharyngitis (1), laryngitis (1), chronic pulmonary emphysema (2) and pulmonary abscess (1). The most prominent alterations in the blood concerned the proteins, which in three of the cases exceeded 7 Gm. per 100 cc. of total serum protein. In six cases the A:G ratio was less than 0.9. These changes were attributed to inflammatory reaction, which promotes the production of globulins. At the same time there seemed to be a distinct tendency toward low calcium (table IX). Phosphorus apparently was unaffected; in some cases it was increased sufficiently to compensate for the reduced Ca^{++} in the calculated Fv.

The more advanced cases in this group, in which the damage involved deep parts of the respiratory tree, showed more marked changes in the proteins and lower calcium figures than milder cases. In the former the tendency was toward normal phosphorus and low Fv. The extent of improvement in these cases to be derived from calcium therapy, alone or in combination with phosphorus, is problematic; the results obtained thus far from a few cases have been encouraging but not definite.

Gastrointestinal.—Of this group nine were cases of diarrhea, either intermittent or continuous; one a case of recurrent colic, considered to be of intestinal origin; one of serious constipation, and one of heavy strongyle infestation. The most striking change noted in the series of diarrhea cases was the frequence of hypophosphatemia, which occurred in eight of the nine studied. A slight reduction in calcium was equally prominent in this series, but the extent of this reduction was not nearly as great as that of phosphorus. Table IX reveals that eight of the twelve cases were low in Ca^{++} and nine were low in phosphorus, but the average reduction below the normal minimum was 0.2 mg. for Ca^{++} and 0.5 mg. for phosphorus. These reductions are proportionately equal, on the bases of the coefficients of the terms in the coördination equation, *e.g.*, $0.2 \times 1\ (Ca^{++}) = 0.5 \times 0.4\ (P)$.

These effects presumably result from the accelerated loss of calcium and phosphorus in the stools due to the diarrhea. The single case of constipation included in this series was normal in all these respects. Serum proteins were inclined to be somewhat increased, especially where the diarrhea was severe, so that the total calcium levels were not diminished as frequently as Ca^{++} (table IX). Only one case showed a total calcium less than 11 mg. per 100 cc. The other three were but slightly below the minimum of 11.7 used for reference in this study (see table I; mean Ca 12.2 ± 0.5). In one case (No. 154) the total calcium was high, but in the presence of the high protein level indicated, Ca^{++} was just below the normal minimum of 5.8 mg. per 100 cc.

Protein fractionation suggested three distinct types of gastrointestinal cases. The benign cases were characterized by a normal A:G ratio; more severe cases, which eventually responded favorably, although in some instances after a long period, showed a low ratio; the two cases of fatal enteritis (Nos. 104 and 168) had high A:G ratios. Although calcium and phosphorus therapy was used on both these last, and No. 168 showed a response in the blood findings, the outcome was the same. Case

TABLE IX—Summary of Findings in Respiratory, Gastrointestinal and Nervous Cases

Cases	Ca			Ca^{++}			P			Fv			Averages "Low" Groups*		
	Low	s. d.†	High	Low	s. d.†	High	Low	s. d.†	High	Low	s. d.†	High	Ca^{++}	P	Fv
Respiratory (10)	8	4	1	10	3	0	1	9	3	6	7	0	—0.7	—0.2	—0.8
Gastrointestinal (12)	4	6	2	8	3	1	9	3	0	8	4	0	—0.2	—0.5	—0.9
Nervous (15)	7	8	4	6	11	2	4	11	4	6	11	2	—0.4	—0.7	—0.8

*Averages of findings obtained in the cases deficient according to the s.d. ranges; results expressed as mg. per 100 cc. serum (Ca^{++} and P).

†Numbers of cases falling within the various groups, as in table VII.

TABLE X—Typical Records from Respiratory, Gastrointestinal, Nervous and Miscellaneous Cases

No.	Age	Date	Condition	Ca (Mg. %)	Ca^{++} (Mg. %)	P (Mg. %)	Fv*	Proteins (Gm. %)	A:G
	Respiratory								
143	7 yr.	10–21–38	Chronic pulmonary emphysema ("heaves")	12.1	*5.6*	3.0	—0.4	*7.7*†	*0.5*
114	14 yr.	8–31–38	Chronic pulmonary emphysema	*10.4*	*4.7*	3.5	—1.1	*7.3*†	*0.8*
115	14 yr.	8–31–38	Chronic bronchitis	12.1	*5.3*	3.6	—0.5	*8.1*†	*0.6*
	Gastrointestinal								
154	1½ yr.	10–28–38	History of scouring; weak; colicky	*13.0*†	*5.7*	*3.8*	—0.9	*7.7*†	
101	4 mo.	8–16–38	Scouring for past 2 days	*12.8*†	6.3	6.8	0	6.4	1.5
		11– 9–38	Scouring now severe	12.2	*5.5*	*3.0*	—2.0	6.8	*2.0*†
		11–16–38	Died; hemorrhagic enteritis						
168	4 yr.	12– 3–38	Poor general condition; frequent attacks of diarrhea	*11.5*	*5.6*	*2.5*	—0.8	6.2	
		1–30–39	Has been out on pasture; improved	12.5	6.1	3.2	0	6.4	
		10– 3–39	Appears in fair condition	12.3	6.1	2.7	0	6.0	*2.1*†
			Died; strangulated hernia of bowel						
	Nervous								
59	3 yr.	4–26–38	Tetany of facial and leg muscles since birth; moves stiffly	*11.3*	5.8	3.4	—0.4	5.6	1.4
		9–27–38	Symptom less marked, but present	11.9	*5.5*	3.8	—0.5	*7.0*†	1.1
		7– 8–40	Symptom almost gone; still twitches at nostrils	12.0	6.4	2.7	+0.2	5.3	
241	14 mo.	6– 8–39	General incoordination; no improvement under treatment	12.5	6.1	*3.4*	—0.7	6.4	1.1
245	10 yr.	7–12–39	Severe posterior incoordination; pregnant	12.1	5.5	*1.4*	—1.1	7.3	0.9
		7–18–39	Much improved	*13.4*†	*6.2*†	*5.2*†	+1.1	7.2	
		8– 7–39	Normal	*13.6*†	6.1	3.3	+0.2	*6.7*†	0.7
	Miscellaneous								
43	7 yr.	4– 5–38	Azoturia; both hind legs involved	*10.6*	*5.4*	2.7	—0.7	5.7	1.0
145	1 yr.	10–24–38	Polyuria, first attack	12.3	*5.5*	5.3	—0.6	*7.4*†	1.1
		12–13–38	Recovering from second attack	*11.4*	5.8	5.4	—0.2	5.8	
77	6 yr.	5–20–38	Serum horse; marked asthenia‡	*11.5*	*4.9*	*1.6*	—1.7	*8.8*†	*0.3*

*$Ca^{++} + 0.4\ P - C_{age} = Fv$.

†In excess of normal range; figures *in italics* without (†) represent values lower than the normal minimum.

‡Courtesy of the National Drug Company, Swiftwater, Pa.; specimen from one of the laboratory subjects.

104 failed even to indicate any response to the treatment.

Nervous.—These are cases in which the symptoms point to functional disturbances in the absence of demonstrable anatomic change. They include cases of incoördinated gait (5), psychic conditions described as "nervousness" accompanied by symptoms of reduced general vitality (5), tetany-like cases (2) and others to a total of 15, on which 19 examinations were made.

The causal relationship between hypocalcemia and the symptom complex, tetany, first demonstrated by McCallum and Voegtlin[62] in 1909, suggested the possibility of low blood calcium in some of the cases in this group. Under normal conditions of serum-protein content and pH of the blood, tetany would not be expected until the total calcium in the blood is reduced to approximately 7 mg. per 100 cc. of serum. The lowest total calcium encoun-

[62]McCallum, W. G., and Voegtlin, C.: On the relation of tetany to the parathyroid glands and to calcium metabolism. J. Exp. Med., xi (Jan. 1909), p. 118.

tered in this group was 10.7, in the presence of a normal protein level (No. 281), a case of "nervousness" with diminished appetite and poor general condition. The two cases with symptomatic tetany (Nos. 59 and 80) hardly conform to the classical blood picture of hypocalcemic tetany.

It is difficult to draw general conclusions regarding the diagnostic value of these tests in cases of this order. In two instances, however, a marked hypophosphatemia was encountered associated with incoördination. One of these, a 14-month-old colt (No. 241), had a history of sudden loss of its ability to move about normally, finally becoming unable to stand at all. Psychic reactions appeared normal. Despite treatment with phosphates, vitamin D concentrates and a very good diet (appetite was normal), it failed to improve and finally was destroyed. The other subject, a mare in foal, rapidly became incoördinated in the hind legs and finally was unable to walk. Oral use of phosphates (Na_2HPO_4) was attended by a prompt return of normal function with concomitant rise in the blood phosphorus (No. 245).

Miscellaneous.—From this group there is little to be concluded, since the cases are symptomatically diverse. In one subject a prolonged siege of polyuria was accompanied by a rise in serum-protein level (No. 145). Upon recovery the proteins returned to normal, with a parallel fall in total calcium. Ca^{++} actually showed a slight increase between the two analyses, although Fv was within normal limits on the first examination.

Another case, a horse which had been bled periodically for serum production, showed low protein and phosphorus levels.

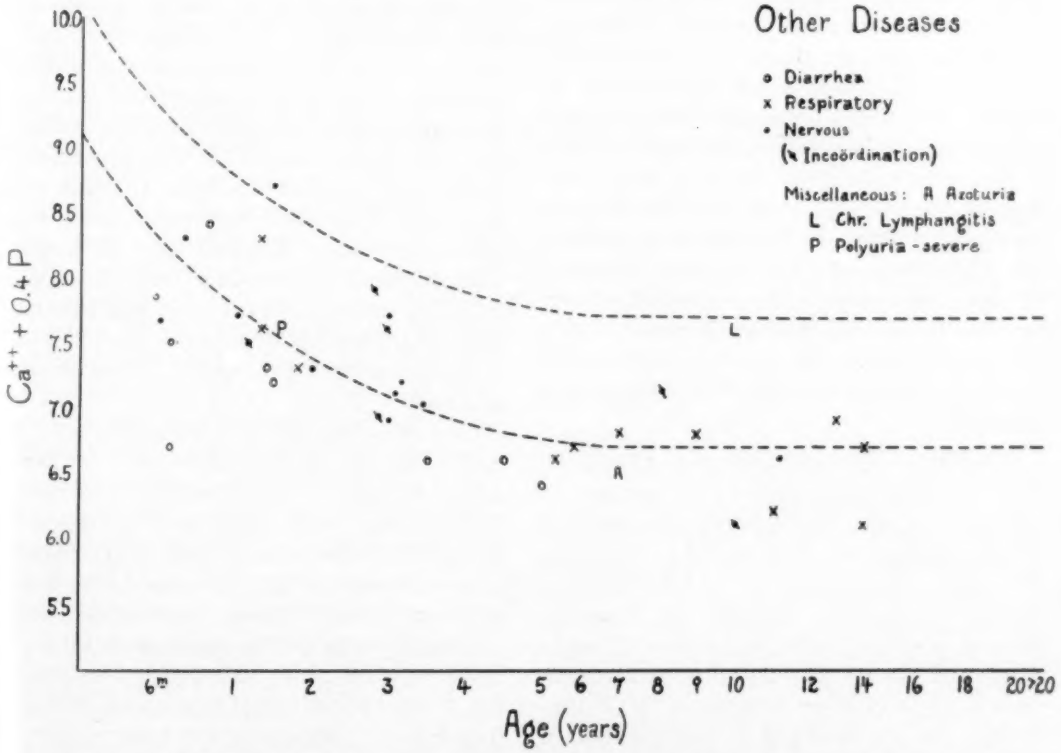


Fig. 6. Distribution of results from cases with disturbances involving the soft tissues. These cases are subdivided on an anatomic-physiologic basis, as illustrated by the data in tables IX and X. No case with bone lesions is included. The results indicate that gastrointestinal symptoms (diarrhea) are associated frequently with marked reductions in Fv. Respiratory cases are inclined to be low normals, if not distinctly subnormal in this respect. The findings in nervous cases lack uniformity, some being normal and others distinctly low. Other cases yielded scattered results.

Although total calcium was somewhat reduced, Ca^{++} was normal, the low protein accounting for the difference. Two other cases, which had been hyperimmunized against tetanus and bled periodically for antisera, showed pictures which might be considered extreme cases of general reaction to infection (No. 77). Total serum proteins were elevated enormously in these cases, with marked reduction in the A:G ratio; the albumin level was about half the normal average, and globulins were increased approximately four times. Since total serum calcium showed slight reduction, Ca^{++} was depressed considerably. Phosphorus also was unusually low, so that the Fv results were among the lowest encountered in the entire series.

This is a typical, but extreme, illustration of the trend in cases of a general inflammatory nature. It was manifest in some of the cases included in the preceding groups. The question naturally arises as to whether the apparent reduction in Ca^{++}, as calculated from the normal or slightly diminished total calcium and high protein figures, should be regarded as real. It is conceivable that changes may take place in the proteins, such as a rise in fractions with a more limited power of combining with calcium than is now assumed. On the other hand, the beneficial effects ascribed to calcium therapy in cases of this nature support the original conception. Further evidence is needed to answer this question.

Bleeders.—Veterinarians are familiar with the occasional race horse subject to recurrent nosebleed, in which no evidence of local damage can be detected in the nasal structure. During an attack, bleeding occurs repeatedly, varying in intensity from a scanty trickle to profuse hemorrhage and always associated with vigorous exercise. During an attack, it is characteristic for the bleeding to begin either in the course of or immediately after finishing a race or brisk exercise run. The typical case shows repeated bleeding, in some instances becoming progressively more severe for a while, to be followed by gradual recession. In other cases the bleeding is less frequent, but the tendency persists for a longer period. In all instances the hemorrhage stops spontaneously after periods ranging from a few minutes to a half hour or more.

The present series includes five cases of spontaneous epistaxis, reviewed in table XI. Only one of these animals (No. 23) was studied by repeated blood examinations. The course of this case was essentially that of the others. At the time of the attacks her general condition was not altered to an appreciable extent; in the opinion of the attendants she appeared somewhat sluglish and less vigorous than usual. There was a preliminary period of general dullness lasting several days, during which her endurance seemed diminished and she failed to attain her usual speed on the track. At this time the attendant noticed a slight edema ("filling") in the upper neck and throat region. This was followed a few days later by the appearance of a small amount of bloody discharge from the nostrils during and following the daily exercise run. As days passed, this became progressively more marked until the bleeding was profuse. The only treatment used was calcium gluconate, intravenously and orally, each day until the bleeding failed to appear on brisk exercise. Recovery was gradual, with improvement in general condition accompanying the diminution in daily loss of blood.

Clinical observation has suggested the possibility of calcium deficiency in these cases. Colonel C. W. Greenlee, in a recent conversation with one of the writers, pointed out that this condition is common in race horses of the Panama Canal Zone, and has been treated successfully with calcium therapy. The implication that the blood calcium is so deficient as to retard the formation of thrombin seems without foundation. Although we have made no careful determinations of clotting time, casual observation indicates no gross abnormality in coagulation. Furthermore, the lowest blood-calcium levels obtained on the cases we have examined are far in

TABLE XI—Cases of Spontaneous Epistaxis

No.	Age (Yr.)	Date	Condition	Ca (Mg. %)	Ca++ (Mg. %)	P (Mg. %)	Fv	Proteins (Gm. %)	A:G*
23	8	2–15–38	Normal	*14.1*†	*6.9*†	2.7	+0.8	6.3	1.6
		8– 5–38	Normal; racing well	*11.4*	5.9	*5.1*†	+0.7	5.6	1.7
		9–14–38	Bleeding slightly on exercise	*11.5*	5.9	3.1	0	5.6	1.8
		10– 3–38	Bled considerably on racing	*10.9*	*5.6*	3.2	−0.3	5.3	
		10–10–38	Stopped bleeding	12.6	6.3	3.5	+0.5	5.9	1.8
		2–12–39	Normal	*13.8*†	*6.8*†	2.9	+0.8	6.2	
		3–20–39	Dulness; "filling in throat"‡	*12.8*†	*6.6*†	2.7	+0.5	5.5	
		5– 8–39	Bleeding slightly on exercise	12.4	6.4	?	?	5.5	
99	17	7–15–38	Bleeding 6 months ago, and recently more severely	12.2	6.1	*3.8*†	+0.4	6.2	1.0
122	7	9– 6–38	"Bleeder"; not bleeding lately, but "filling in throat" observed	11.7	*5.6*	*4.1*†	0	6.4	1.1
133	5	10– 3–38	Small amount of blood lost after each race for past year	*11.6*	6.0	3.3	0	*5.4*	1.8
254	4	10– 4–39	Bleeding on exercise and "filling in throat"	*13.1*†	6.3	2.9	+0.1	6.4	

*$Ca^{++} + 0.4\ P - C_{age} = Fv$.

†In excess of normal range; figures *in italics* without (†) represent values lower than the normal minimum.

‡A mild edema of the upper neck and throat region; in horses which intermittently show attacks of repeated epistaxis, this edema frequently precedes the actual bleeding by several days.

? = determination unsatisfactory.

excess of the concentrations limiting coagulation.[63-65] If calcium deficiency is a factor in this syndrome, the mechanism remains obscure.

It is interesting to note that at the time of bleeding the blood calcium and phosphorus were not low, considered both individually and coördinated as Fv, although low normals were observed for Ca^{++} and Fv when the bleeding was most severe (No. 23). In this particular subject the levels were unusually high during the normal period, showing a gradual decline with the approach of the time when bleeding was most severe. These results perhaps may be interpreted to mean that this animal normally has higher calcium and phosphorus levels than the average horse, the reductions actually leading to distinctly subnormal values in this case; but since all the other animals in this group gave normal blood findings at the time of the epistaxis and before treatment was begun, and since many horses have been found with much lower calcium and phosphorus levels yet no history of nosebleed, we are inclined to question this explanation. In view of these results, it is difficult to believe that the blood calcium is affected primarily in these cases.

Many cases of minor disturbances have been encountered in which the blood findings were within the normal range. These include cases manifesting intestinal parasitism, temporary periods of inappetence and unthriftiness, and other symptoms common among Thoroughbreds. In view of the lack of alteration in the blood composition, these cases are not discussed in this review.

Discussion

Without venturing a review of the vast literature on the maintenance and regulation of the blood calcium and phosphorus, the writers suggest the following as an arbitrary working concept of the principal factors involved. Primarily the blood is supplied with calcium and phosphorus by intestinal absorption of these elements at a rate determined by their concentration and availability in the food. At the same time a large part of the fraction absorbed is being eliminated through the kidneys and the intestines. The difference between the quantities absorbed and eliminated determines the balance, a positive balance indi-

[63] Ferguson, J. H.: The blood calcium and the calcium factor in blood coagulation. Physiol. Rev., xvi (Oct. 1936), p. 640.

[64] Crane, M. M., and Sanford, H. N.: The effect of variations in total calcium concentration upon the coagulation time of blood. Amer. J. Physiol., cxviii (April 1937), p. 703.

[65] Ransmeier, J. C., and McLean, F. C.: The relationship of calcium ion concentration to the coagulation of citrated plasma. Amer. J. Physiol., cxxi (Feb. 1938), p. 488.

cating retention of the elements in the body and a negative balance indicating demineralization. At the same time the blood is in contact with the massive stores of calcium and phosphorus precipitates, the bones. During active ossification the skeleton withdraws these elements from the blood and deposits them as salts in the bone structure. Conversely, during decalcification the skeleton throws off calcium and phosphorus into the blood at the expense of its own substance.

The coexistence, as has been pointed out, of Ca^{++} and PO_4 in the concentrations usual in blood, in direct and continuous contact with the precipitated salts of the bone (predominantly phosphates and carbonates of calcium), is difficult to interpret on the basis of chemical solubility alone. The principal theories suggested to explain this inconsistency have been reviewed ably.[45, 46] It seems to us that the most acceptable current explanation assumes that the excess ions, beyond what may be accounted for on a molecular dissociation basis, are maintained in equilibrium with the precipitates of bone by a special control. Parathyroid activity is charged with this control. Possibly to a minor degree, vitamin D and thyroid hormone have a similar function. The phenomenon is known as "biologic solubility."

According to the data summarized in figures 3 and 4, variations may occur between the blood calcium and phosphorus levels without affecting the value of Fv. When marked reductions do occur in the combined value of these elements, as indicated by a low Fv, it follows that either a reduction in the "biologic solubility" has occurred (*e.g.*, diminished parathyroid activity), or the supply of one or both the elements is inadequate to maintain the concentration permitted. Moreover, it is logical to conclude that when such a deficiency exists, the deficient blood is less in a position to supply calcium and phosphorus to the bones and more likely to remove these elements from the skeleton than when the blood contains the normal quantities. Therefore, if it can be assumed that the low level of calcium and phosphorus in the blood, as measured by the formula suggested, is not the result of reduced "biologic solubility," the chances are fair that it is associated with progressive decalcification of the bones.

Our findings on the cases with bone lesions may be interpreted in terms of this concept. The acute cases showed distinct deficits in both Ca^{++} and phosphorus. Reduction in phosphorus was striking, although total serum calcium scarcely could be considered other than low normal. These observations are in general agreement with the findings in bone weakness of other species. Rickets and osteomalacia, affecting man and the laboratory animals, are characterized by reductions in either calcium or phosphorus, occasionally in both. Hypophosphatemia, with a normal or low calcium, is the more common finding in rickets of children, but hypocalcemia combined with normal blood phosphorus sometimes is encountered. On the other hand, osteomalacia in adults is associated more commonly with low calcium, although deficient serum phosphorus has been reported frequently. In this connection, we would point out that the subacute cases in the series of horses studied showed a definite tendency toward low Ca^{++} in the presence of normal phosphorus. The same tendency in the chronic cases was to a large measure offset by the relative increases in blood phosphorus.

An important question arises as to whether mechanical injuries to the bones (*e.g.*, splints, spavins, etc.) are to a greater degree the result of poor conformation or structural bone weakness. Obviously, defects in conformation which lead to unusual strain on any part of the skeleton of the horse may account for bone lesions. Even in those with faultless conformation, a sudden strain, as a wrench in making a quick turn or on losing balance, may result in the same lesions. Why, then, do these lesions occur in some horses and not in others subject to similar strains? In feeding studies on race horses, Humphrey[30] noticed a higher incidence of bone defects in horses on rations low in calcium

and phosphorus than was found when the ratio was improved. In our experiences with horses on different farms, we observed the same varying incidence of bone lesions between farms where the mineral supply was at different levels. On farms where calcium and phosphorus were abundant, bone lesions were not nearly as common in the horses; and blood findings were higher, particularly calcium, than on farms less favorably supplied. It seems logical to conclude that an adequate supply of calcium and phosphorus is required to assure maximum strength of bone. Moreover, the blood content of calcium and phosphorus provides a ready index to the adequacy of this supply.

Where the pathologic processes involved soft tissues, without apparent effect on the bones, the calcium and phosphorus variations in the blood in most cases could be interpreted as influences of hydration and inflammatory reaction. Variations in the water content of blood, as in the dehydration of purging and polyuria, are attended by altered concentrations of blood solids. Dehydration results in a rise in the serum protein, without change in the A:G ratio in uncomplicated cases. In conjunction with this rise in proteins there is an increase in total calcium, in cases where Ca^{++} remains constant. With the purging of diarrhea, however, considerable calcium and phosphorus are lost, resulting in definite reductions in the Ca^{++} and phosphorus levels in the blood.

Infections, and other conditions in which a general inflammatory reaction develops, are characterized by a rise in serum globulin, and later a fall in albumin. The A:G ratio is reduced proportionately. In this series, these effects are most marked in the horses hyperimmunized against tetanus, but they are apparent to a varied degree in many of the respiratory and gastrointestinal cases. As indicated, the A:G ratio is normally lower in pregnant and postparturient mares than in males and nonpregnant females; but where this ratio is reduced significantly from the normal (*i.e.*, less than 0.9 in nonpregnant animals) a general inflammatory reaction is indicated. It may be considered as a basis for speculation that the elevated A:G ratios in the two fatal cases of enteritis (described in the preceding section), indicating reduction in the serum globulin, are evidence of impending failure in the physiologic defense mechanism: asthenic collapse.

Ca^{++} tends to be diminished in conjunction with inflammatory reactions in serum proteins. Total calcium findings are usually normal or low normal, the reductions in Ca^{++} manifesting a secondary influence of hyperproteinemia. It must be kept in mind, however, that the calculated value for Ca^{++} depends on a constant calcium-binding power of the serum proteins. Whether or not this power remains constant under conditions of the protein alterations encountered is open to question. If the calcium-binding power of the proteins is less than is assumed from the studies of Van Slyke, Hastings, Hiller and Sendroy, then it follows that the Ca^{++} reductions are also less. The tendency toward high normal phosphorus levels in less severe cases of this group, in which normal Fv values are found, suggests that a reaction takes place in phosphorus secondary to the reductions in Ca^{++}.

Our results indicate little change in blood calcium and phosphorus attendant upon cases with symptoms of tetany. Similarly, horses with a tendency toward spontaneous epistaxis (bleeders) show no abnormality. Acute hypophosphatemia, attended with normal blood calcium, was encountered in two cases of muscular incoördination. These cases were described in the section on nervous disturbances.

Effects of Therapy.—While the course of treatment was not the same in all cases, the objective was always to correct any calcium and phosphorus deficit in the blood, especially where associated with a low Fv value (less than —0.5). Various therapeutic agents were employed to this end, and in a variety of dosages. Those used most frequently were calcium gluconate (orally and intravenously), Na_2HPO_4 (orally), bone meal (in feed), and proprietary vitamin D products. The effect of

these agents was always to increase the blood concentration of the element supplied, the degree in proportion to the extent of the former deficit. For example, in a case with low Ca^{++}, the increase upon augmenting calcium intake was greater than where Ca^{++} was normal. Furthermore, little effect was noted in cases with high Ca^{++} levels, even when comparatively large amounts of calcium were administered. Manifestly, it is not difficult to augment the blood calcium and phosphorus therapeutically when these elements are deficient.

The important question, however, concerns the ultimate therapeutic value of this treatment. Our observations lead us to feel that calcium and phosphorus therapy is of definite value in promoting recovery in acute and subacute bone lesions, in cases manifesting blood deficits in these elements. The value of this treatment in chronic cases is subject to dispute. From the therapeutic point of view, bone lesions may be divided into two groups (1) those with active inflammation of the bone (acute and subacute); and (2) those inactive but identified with the accumulated products of previous active lesions (chronic). Accordingly, the probable value of calcium and phosphorus therapy in any case of inflammatory bone lesion would seem to depend on the degree of activity in the inflammation, and to the extent it is associated with blood deficits.

In cases other than those involving bone disease this therapy has been tried over a range of diagnoses. The results in the majority of cases failed to associate striking improvement with the addition of calcium and phosphorus. Individual cases, however, seemed somewhat improved by this measure, particularly some of those classed as respiratory. Whether or not this therapy was of any value in the majority of gastrointestinal and nervous cases is a matter of speculation; certainly, the results were not striking. An exception was encountered in a mare with posterior incoördination, discussed previously, which responded promptly to phosphates.

SUMMARY

1) Among Thoroughbred owners the practice of training and racing young horses necessitates early development of strong bone. Bone lesions are frequent in these young animals, often leading to permanent unsoundness and, at least, temporary loss of their services. There is much reason to suspect a connection between bone weakness and deficiency of calcium and phosphorus in the blood. It has been indicated also that a deficiency of these elements may be associated with low vigor and poor general condition, especially in young horses.

2) In this study total serum calcium ranged normally from 11 to 13.5 mg. per 100 cc., averaging 12.2 (s.d. 0.5). Variations within this range could be associated with seasonal change and differences in the feeding, but no influence could be ascribed to age and sex. Pregnant mares, especially during the advanced stages of pregnancy, showed a tendency toward reduction in total calcium.

3) The age of the horse appears to be the most important factor in determining the blood level of inorganic phosphorus. Findings varied grossly from 2 to 8 mg. per 100 cc. of serum, receding progressively from a maximum in foals less than 3 months old to the minimum levels of maturity. Also, results obtained at different times of the year indicate a seasonal tide similar to that observed by Hess and Lundagen in children; but in this study the effect was not restricted to young animals. Sex, pregnancy and differences in feeding did not appear in this series to be significant factors in determining the blood phosphorus.

4) Total serum proteins varied normally from 5 to 7 Gm. per 100 cc., averaging 6.1 (s.d. 0.3). Age, seasonal change and pregnancy induced variations within this range. In fact, under the influence of normal pregnancy the level of total protein frequently exceeded 7 Gm. per 100 cc. Sex differences had no bearing on protein levels, nor did the variations in feeding encountered in this survey.

From a study of the A:G ratio it was evi-

dent that the variations in total protein were due largely to changes in the globulin fraction. Young foals, in which the lowest protein levels were encountered, revealed the highest A:G ratios; pregnant mares showed very low A:G ratios. Lack of significant variation in the A:G ratio at different times of the year suggests that the seasonal trends in total protein level (described in figure 1) are in the nature of a dilution of the serum in the spring and fall, with a subsequent concentration during the intervening periods.

5) The relatively wide ranges of normal variation observed in serum calcium and inorganic phosphorus in a large measure overlap the findings reported for cases of recognized abnormality. Since these variations in calcium and phosphorus are to a significant extent the result of interactions among calcium, phosphorus and protein, a method was sought for coördinating the results of the three analyses run simultaneously, as an aid in identifying primary disturbances in the blood calcium and phosphorus. To this end the writers propose the following equation, in which Ca^{++} is calculated from the figures for total Ca and protein, according to the formula of McLean and Hastings:

$$Ca^{++} + 0.4\ P - C_{age} = Fv.$$

In this equation Ca^{++} and phosphorus (inorganic) are expressed as milligrams per 100 cc. of serum; C_{age} is a constant varying with the age of the horse (table IV); and Fv, the result, is normally zero, with a standard deviation of 0.3.

6) Cases with acute bone lesions (*e.g.*, acute arthritis, fracture) revealed a high incidence of blood calcium and phosphorus deficiency, with a reduction in phosphorus quantitatively the greater. In subacute cases (*e.g.*, subacute periostitis and ostitis), however, these effects were progressively less pronounced, apparently varying with the degree of active inflammation present in the individual cases. Chronic bone lesions (*e.g.*, ringbones, spavins and other exostoses of chronic nature) rarely were associated with calcium and phosphorus deficits in the blood. In fact, the averages for this group approximated those for normal horses.

7) From the study of clinical subjects with diseases of the soft tissues, a distinct tendency toward slight hypocalcemia was observed in respiratory cases (*e.g.*, bronchitis, pulmonary emphysema), and diarrhea was associated with marked reduction in the blood levels of both calcium and phosphorus. On the other hand, hypocalcemia never was encountered in connection with symptoms of tetany and various other manifestations of nervous instability, although low phosphorus was observed in two cases with muscular incoördination. The results from examinations of horses with recurrent spontaneous epistaxis (bleeders) failed to reveal any basis for attributing the syndrome to calcium deficiency.

ACKNOWLEDGMENTS

The studies here reported would not have been possible without the enthusiastic coöperation of a group of prominent Thoroughbred breeders of Maryland. The writers wish to acknowledge the help generously provided by this group in permitting the use of their animals and in supplying assistance in the form of records, and in some instances special procedures necessitated by the demands of particular experiments. Also, the writers are indebted to the clinical staff of the Veterinary Hospital, University of Pennsylvania, for coöperating with clinical cases; to several veterinarians in private practice, particularly R. L. Booth of Middleburg, Va., and E. A. Rile of Blue Bell, Pa.; to R. S. Amadon and J. H. Jones of the faculty of the School of Veterinary Medicine, University of Pennsylvania, for helpful suggestions throughout the course of this project; to J. G. Reinhold and his staff in the laboratory of the Philadelphia General Hospital, for assistance in developing the analytic methods for serum-protein determinations; and to F. C. McLean of the University of

Chicago, for supplying copies of his charts dealing with the blood-calcium problem.

Finally, it is the wish of all concerned with the work of this project to express appreciation to the two benefactors, prominent Thoroughbred breeders who wish to remain anonymous, whose gifts have been the material support of this study.

Announcement

The Division of Veterinary Medicine, University Farm, St. Paul, Minn., announces the recent translation of the following articles which may be of interest to others in the profession. This work was done by translators working on the campus under the Work Projects Administration, with technical supervision by members of the staff of the Division of Veterinary Medicine. It is listed as official project No. 65-1-71-140, sub-project No. 474.

In addition to the copies which remain in the Division's files, one copy of each translation has been filed with the American Documentation Institute, Washington, D. C., in order to make this material available to research workers in other fields.

Lousse, A.: "Un cas de brucellose équine à localisation sternale." Annales de Médecine Vétérinaire, lxxxii (1937), pp. 413-416.

Santagostino, Carlo: "La mastite acuta parenchimatosa delle bovine da germi Coli-aerogenes." La Clinica Veterinaria, lxi (1938), pp. 523-531.

Vianello, Gino: "Le mastiti infettive dei bovini." *Ibid.*, pp. 201-208.

Ibid.: "Un'enzoozia da mal rossino nei fagiani." *Ibid.*, pp. 234-245.